Université de Montréal

# Development of an enzyme immobilization platform based on microencapsulation for paper-based biosensors

par

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Thèse présentée à la Faculté des études supérieures et postdoctorales

en vue de l'obtention du grade de *Philosophiae Doctor* (Ph.D)

en Chimie

November, 2011

Université de Montréal

Faculté des études supérieures et postdoctorales

Cette thèse intitulée :

# Development of an enzyme immobilization platform based on microencapsulation for paper-based biosensors

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# Résumé

Un papier bioactif est obtenu par la modification d'un papier en y immobilisant une ou plusieurs biomolécules. La recherche et le développement de papiers bioactifs est en plein essor car le papier est un substrat peu dispendieux qui est déjà d'usage très répandu à travers le monde. Bien que les papiers bioactifs n'aient pas connus de succès commercial depuis la mise en marche de bandelettes mesurant le taux de glucose dans les années cinquante, de nombreux groupes de recherche travaillent à immobiliser des biomolécules sur le papier pour obtenir un papier bioactif qui est abordable et possède une bonne durée de vie. Contrairement à la glucose oxidase, l'enzyme utilisée sur ces bandelettes, la majorité des biomolécules sont très fragiles et perdent leur activité très rapidement lorsqu'immobilisées sur des papiers. Le développement de nouveaux papiers bioactifs pouvant détecter des substances d'intérêt ou même désactiver des pathogènes dépend donc de découverte de nouvelles techniques d'immobilisation des biomolécules permettant de maintenir leur activité tout en étant applicable dans la chaîne de production actuelle des papiers fins.

Le but de cette thèse est de développer une technique d'immobilisation efficace et versatile, permettant de protéger l'activité de biomolécules incorporées sur des papiers. La microencapsulation a été choisie comme technique d'immobilisation car elle permet d'enfermer de grandes quantités de biomolécules à l'intérieur d'une sphère poreuse permettant leur protection. Pour cette étude, le polymère poly(éthylènediimine) a été choisi

afin de générer la paroi des microcapsules. Les enzymes laccase et glucose oxidase, dont les propriétés sont bien établies, seront utilisées comme biomolécules test. Dans un premier temps, deux procédures d'encapsulation ont été développées puis étudiées. La méthode par émulsion produit des microcapsules de plus petits diamètres que la méthode par encapsulation utilisant un encapsulateur, bien que cette dernière offre une meilleure efficacité d'encapsulation. Par la suite, l'effet de la procédure d'encapsulation sur l'activité enzymatique et la stabilité thermique des enzymes a été étudié à cause de l'importance du maintien de l'activité sur le développement d'une plateforme d'immobilisation. L'effet de la nature du polymère utilisé pour la fabrication des capsules sur la conformation de l'enzyme a été étudié pour la première fois.

Finalement, l'applicabilité des microcapsules de poly(éthylèneimine) dans la confection de papiers bioactifs a été démontré par le biais de trois prototypes. Un papier réagissant au glucose a été obtenu en immobilisant des microcapsules contenant l'enzyme glucose oxidase. Un papier sensible à l'enzyme neuraminidase pour la détection de la vaginose bactérienne avec une plus grande stabilité durant l'entreposage a été fait en encapsulant les réactifs colorimétriques dans des capsules de poly(éthylèneimine). L'utilisation de microcapsules pour l'immobilisation d'anticorps a également été étudiée.

Les avancées au niveau de la plateforme d'immobilisation de biomolécules par microencapsulation qui ont été réalisées lors de cette thèse permettront de mieux comprendre l'effet des réactifs impliqués dans la procédure de microencapsulation sur la

stabilité, l'activité et la conformation des biomolécules. Les résultats obtenus démontrent que la plateforme d'immobilisation développée peut être appliquée pour la confection de nouveaux papiers bioactifs.

**Mots-clés** :

Microencapsulation, microcapsules de poly (éthylèneimine), encapsulateur, buse vibrations, papiers bioactifs, conformation des enzymes, fluorescence, microscopie confocale à balayage laser (CLSM), biocapteur colorimétrique, microcapsules d'anticorps sensibilisés.

# Abstract

Biosensing paper attracts increasing attention due to its benefits of being simple, visible, portable and useful for detecting various contaminants, pathogens and toxins. While there has been no bioactive paper commercialized since glucose paper strips developed in the fifties, many research groups are working to immobilize biomolecules on paper to achieve a bioactive paper that is affordable and has good shelf life. The goal of this research is to develop some highly useful bioactive paper that could, for example, measure blood glucose, or immediately detect and simultaneously deactivate pathogens such as neuraminidase and E.coli. Previously, bioactive paper was produced either through physically absorbing biorecognition elements or printing bio-ink onto paper substrate. Our methodology for fabrication of bioactive paper strips is compatible with existing paper making process and includes three procedures: the fabrication of microcapsules, enzyme or antibody microencapsulation, immobilization of enzymes or antibody-entrapped microcapsules into paper pulp.

The first step, in fabricating of bioactive paper strips is to produce biocompatible and inexpensive microcapsules with suitable parameters. To do so, two types of microencapsulation methods were compared; the emulsion method and the vibration nozzle method accomplished with an encapsulator. The parameters for producing optimal microcapsules with both methods were studied. Factors that affect their diameter, wall thickness, shell pore size, encapsulation efficiency and membrane compositions were also discussed. By comparison, microcapsules prepared with poly(ethyleneimine) (PEI) by the

emulsion method exhibit properties that were more suitable for enzyme encapsulation and paper making process, whereas the microcapsules prepared by the vibration nozzle method were too big to be immobilized within paper pulp, and had lower encapsulation efficiency, enzymatic activity and productivity. Thus the emulsion method was chosen for subsequent experiments such as enzyme and antibody microencapsulation and bacterial vaginosis (BV) paper preparation. Microcapsules made by the emulsion method were semi-permeable in that the diffusion of substrate and product molecules were allowed freely across the membranes but the encapsulated enzymes would be retained inside.

Glucose oxidase from *Aspergillus niger* (GOx) and laccase from *Trametes versicolor* (TvL) microcapsules showed high encapsulation efficiency, but the encapsulation process caused a severe decrease in the specific activities of both enzymes. Results from circular dichroism (CD) studies, fluorescence properties, enzymatic activities of free enzymes and Michaelis-Menten behavior demonstrated that the $V_{max}$ decrease for GOx was due to the restriction of diffusion across microcapsule membranes with pore size less than 5 nm. The microencapsulation process improved the thermal stability of GOx but decreased that of laccase.

Bioactive papers were fabricated either by incorporating microcapsules containing different enzymes or empty microcapsules soaked in substrate and enhancer solution into the paper pulp during the sheet making process. Both the GOx and the BV paper strips underwent a color change in the presence of glucose and potassium iodide, and sialidase from *Clostridium perfringens* respectively. Some preliminary studies on antibody

sensitized microcapsules, in which antibody was either encapsulated within the PEI microcapsules or conjugated to its membranes, were also performed.

Our objective was to establish an enzyme immobilization platform based on microencapsulation techniques for paper based biosensors. Even though our current studies only focused on the microencapsulation of two enzymes, TvL and GOx, as well as the bioactive paper preparation, a similar approach can be applied to other enzymes. We believe that this immobilization method can potentially be employed for bioactive paper preparation on an industrial scale.

## Keywords

Microencapsulation, microcapsules, poly(ethyleneimine), encapsulator, vibration nozzle, bioactive paper, enzymes conformation, fluorescence, confocal laser scanning microscope (CLSM), colorimetric biosensor, antibody sensitized microcapsules.

# Contribution of co-author

The present work has been supported financially by the Natural Sciences and Engineering Research Council of Canada (NSERC) through SENTINEL--- A Canadian bioactive paper network. The research has been supervised by Dr. Rochefort, Université de Montréal (Montréal, Canada). Dr. Rochefort is the co-author for the articles that have been published and will be co-author for the manuscripts that have been submitted or in preparation for publication.

## Parts of this work have been published or submitted

1. Yufen Zhang, Dominic Rochefort, Comparison of emulsion and vibration nozzle methods for microencapsulation of laccase and glucose oxidase by interfacial reticulation of poly(ethyleneimine), *Journal of Microencapsulation*, 2010, 27(8), 703-713. (Chapter 4)

2. Yufen Zhang, Dominic Rochefort, Activity, conformation and thermal stability of laccase and glucose oxidase in poly(ethyleneimine) microcapsules for immobilization in paper, *Process Biochemistry*, 2011, 46(4), 993-1000. (Chapter 5)

3. Yufen Zhang, Dominic Rochefort, Characterization and applications of microcapsules obtained by interfacial polycondensation, *Journal of Microencapsulation*, 2012, 29(7), 636-649. (Chapter 3)

4. Yufen Zhang, Dominic Rochefort, Fast and Effective Bioactive Paper for Self-Diagnosis of Bacterial Vaginosis, Lab on a Chip, in preparation. (Chapter 6)

# List of Abbreviations, initials and nomenclature

| | |
|---|---|
| Ab | Antibody |
| ABTS | 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) |
| AFM | Atomic force microscope |
| BCA | Bicinchoninic acid |
| BCIN | 5-Bromo-4-chloro-3-indolyl-α-D-N-acetylneuraminic acid |
| BET | Brunauer-Emmett-Teller |
| BSA | Bovine serum albumin |
| BV | Bacterial vaginosis |
| CD | Circular dichroism |
| CDEMA | 2-chloro-N-(2,6-diethylphenyl)-N-(methoxy methyl)acetamide |
| CFU | Colony forming units |
| CLSM | Confocal laser scanning microscope |
| CV | Coefficient of variation |
| DBS | Dibutyl sebacate |
| DEG | Diethylene glycol |
| DETA | Diethylene triamine |
| DHEA | Dehydroepiandrosterone |
| DSC | Differential scanning calorimetry |
| EA | Elemental analysis |
| E.Coli | Escherichia Coli |

| | |
|---|---|
| EDA | Ethylenediamine |
| EGDE | Ethyleneglycol diglycidylether |
| ELISA | Enzyme-Linked immunosorbent assay |
| FITC | Fluorescein isothiocyanate |
| GOx | Glucose oxidase from *Aspergillus Niger* |
| HAD | 1,6 Hexamethylenediamine |
| HMDA | 1,6-hexamethylenediamine |
| HMW | High molecular weight |
| LMW | Low molecular weight |
| HPVA | Hydrolyzed polyvinyl alcohol |
| HRP | Horseradish peroxidase |
| IPDI | Isophorone diisocyanate |
| L-B plot | Lineweaver-Burk plot |
| MCR | Monocrotophos |
| MDI | Diphenylmethane-4,4'-diisocyanate |
| MF | Melamine-formaldehyde resin |
| NBT | Nitroblue tetrazolium |
| NC | Negative control |
| NMR | Nuclear magnetic resonance |
| OES | Oligoethoxysiloxane |
| OM | Optical microscope |
| OPD | *o*-phenylenediamine |

| | |
|---|---|
| PBS | Phosphate-buffered saline |
| PBST | phosphate-buffered saline with Tween 20 |
| PCM | Phase change material |
| PEI | Poly(ethyleneimine) |
| PPD | *p*-phenylenediamine |
| PTFE | Polytetrafluoroethylene |
| PU | Polyurethane |
| PVA | Poly(vinyl alcohol) |
| SC | Sebacoyl chloride |
| SEM | Scanning electron microscope |
| SMA | Styrene–maleic anhydride |
| TBS | Tris-buffered saline |
| TC | Trimesoyl chloride |
| TCMP | (Trichloromethyl)pyridine |
| TDC | Terephthaloyl dichloride |
| TDI | Toluene-2,4-diisocyanate |
| TDITC | Terephthaloyl diisothiocyanate |
| TEM | Transmission electron microscope |
| TEOHS | Tetradecaethoxyhexasiloxane |
| TGA | Thermogravimetric analysis |
| TMB | 3, 3', 5, 5' –Tetramethylbenzidine |
| TMP | Trimethylolpropane |

| | |
|---|---|
| TPC | Acid dichloride (or Terephthalic acid) |
| TvL | Laccase from *Trametes Versicolor* |
| UF | Urea-formaldehyde |

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# Acknowledgements

I gratefully acknowledge my supervisor Prof. Dominic Rochefort at Université de Montréal, for giving me the opportunity to pursue my doctorate study in his research group. His valuable scientific guidance, constant encouragement and financial support through my whole study were gratefully appreciated. Many of my success are directly attributed to his own zest for success and thirst for knowledge.

Many thanks to Prof. Karen C. Waldron for the many times I stopped in. She never hesitated to stop what she was doing to help me out.

Thanks to all of past and present group colleagues and Dr. Waldron's group members, not only for the occasional assistance they provided on my projects but for their personal stories, group and birthday parties.

Special thanks to Dr. Kevin J. Wilkinson, Dr. Pierre Lavallée, Dr. Julian X. Zhu, Dr. J. Pelletier and A. Schmitzer for the access to their instruments.

I would like to thank my committee members: Prof. Gil Garnier, Julian X. Zhu, and Karen C. Waldron for your advice and support.

I would like to thank my friends, Kayee Lok, Pei Tsu, Michelle Lin and Tina Lin for their encouragements, help and friendship.

My deepest gratitude goes to parent, parent-in-law and my husband for their love, financial support, comprehension.

# Chapter 1 Introduction

## 1.1 General introduction

The aim of this research is to construct an enzyme immobilization platform using microencapsulation methods to make paper-based biosensors. Biosensing paper strips have shown great promise and a vast market potential. They are increasingly gaining interest from researchers and industry due to their benefits of being inexpensive, portable, disposable, fast-responding and environmentally friendly.

The first paper strip biosensor can be traced back to the first invention of paper-based partition chromatography by Martin and Synge in 1941 [1, 2], for which they were awarded the Nobel Prize in Chemistry in 1952. Both the EAWAG Company [3] and Stocker et al. [4] developed paper biosensors for the detection of arsenic in potable water. Stocker [4] devised a process in which sensor cells were dried on paper such that when it soaked in aqueous solution for 30 min a color change would take place. The paper strip sensor developed by the EAWAG team [3, 5] used genetically modified bacteria that produced a blue coloration even at low arsenic concentrations. After incubation with an arsenite-containing water sample, the bacterial cells produce β-galactosidase, whose activity can be visualized by its conversion of a substrate to a blue colored product. Similarly, Struss [6, 7] reported the development of a portable filter-paper-based strip biosensor for the detection of the bacterial quorum by sensing signaling molecules called N-acylhomoserine lactones. Their bacterial cell-based

sensing system was designed using components of the N-acylhomoserine lactone-mediated quorum signaling regulatory system as the recognition elements and β-galactosidase as the reporter protein. These bacterial-sensing cells were then liquid-dried on strips of filter paper. Using β-galactosidase as the reporter allows for visual monitoring of the analyte-induced signal when a colorimetric method of detection is used at the same time. Yu et al. [8] proposed a paper-based chemiluminescence analytical biosensor device as a point-of-care diagnostic method for the simultaneous quantification of glucose and uric acid in artificial urine. Wang et al. [9] impregnated nanotubes mixed with antibodies to detect microcystin-LR in the paper strip when it came into contact with water contaminated with microcystin-LR. In their method, the antibodies begin to squeeze themselves through the nanotubes to bind to the antigens, such that the spreading apart of the nanotubes changes their electrical conductivity: this change is measurable. Khan et al. [10] developed inexpensive, portable paper-based test strips for blood-typing by printing antibodies onto a thin piece of paper. Savolainen et al. [11] likewise fabricated bioactive paper using screen printing, flexo printing and rod coating methods to immobilize enzymes onto paper. Murray et al. [12] investigated the direct adsorption of pitch on pulp fibers as a papermaking strategy and investigated the potential of surface plasmon resonance to quantify the adsorption of polydisperse colloids at the solid-liquid interface.

Some bioactive papers were produced either though physically absorbing biorecognition elements [13] or printing the bioink onto the paper [14]. In this project, the bioactive paper was prepared by incorporating microencapsulated enzymes into the

paper pulp. Given that the characteristics exhibited by poly(ethyleneimine) (PEI) microcapsules prepared by the emulsion method were confirmed to be suitable for the paper immobilization, we therefore continued to use this method to encapsulate enzymes [15]. The scheme of the membrane-forming reaction of PEI microcapsules is represented in Chapter 5.

As far as the authors are aware, there are but few reports on biological encapsulation using the interfacial polycondensation methods, despite its advantage in providing a tight control of the mean diameter of the microcapsules in a fast preparation. Chapter 3 provides a literature review of microencapsulation technology by interfacial polycondensation, as well as the characterization and applications of produced microcapsules. To our knowledge, no publication so far has been devoted to the nature of the interactions between the microencapsulated enzymes and the membranes of PEI microcapsules. In this study, PEI microcapsules were prepared by a microemulsion method and vibration nozzle method to entrap two different enzymes, glucose oxidase (GOx) from Aspergillus Niger and laccase from Trametes versicolor (TvL), with a high entrapment efficiency [15]. The activities of both enzymes were investigated throughout the various steps of the encapsulation procedure to give insight that may explain the loss of any observed enzymatic activities.

Immobilization of enzymes has invariably resulted in a loss of activity. The loss of enzymatic activity upon immobilization is due to the conformational changes in the enzyme resulting from several complex interactions between protein and polyelectrolytes, such as Lifshitz-van der Waals forces, dipolar or hydrogen bonding,

conformational entropy, electrostatic forces, Coulomb and hydrophobic dehydrations [16, 17]. Additionally, the diffusion limitation of substrate and product molecules for enzymes immobilized inside the microcapsules may be another factor in the apparent reduction of enzymatic activities. The determination of the exact cause of enzymatic activity decrease was therefore a key consideration in the development of our immobilization platform based on microencapsulation.

Takashi et al [18] used polyamide microcapsules prepared by an interfacial polycondensation method to entrap trypsin (I). They observed that the $K_M$ and $V_{max}$ values were considerably lower than those of free trypsin in solution.

The layer-by-layer microencapsulation method has often been used to encapsulate enzymes [19-23], notwithstanding its drawbacks of time-consuming multiple deposition steps as well as the tedious cleansing of particles required after each deposition to remove the excess non-absorbed polyelectrolytes [24]. Another drawback is the necessity of working at very low particle concentrations due to a high tendency for polyelectrolyte-induced particle flocculation, leaving most enzymes and polyelectrolytes lost during the absorption and deposition procedures. Furthermore, this technique requires templates at all times whereby the template removal step results in a decrease in enzymatic activity.

The preparation of bioactive paper requires a high quantity of microcapsules that are strong enough to withstand the mixing or coating process. Moreover, its components including polymers, crosslinker and membranes must be biocompatible with the encapsulated active agents, hence the microencapsulation procedure and the

properties of the materials of choice determine the activity of the encapsulated enzymes.

PEI is a polymer often used to help in the immobilization of biomolecules. For example, it has been utilized to make self-assembled catalase/PEI-PEG (polyethylene glycol) complexes [25], and “nanozymes” to treat Parkinson’s disease by selectively delivering antioxidants to the substantia nigra pars compacta (SNpc) to attenuate the oxidative stress, thereby increasing the survival of dopaminergic neurons. In another example, the co-aggregation of glutaryl acylase with the PEI system favored the cross-linking between the very reactive and abundant primary amino groups of the PEI and the primary amino groups on the enzyme surface and as such, PEI was used to prevent the release of enzymes from the aggregate [26]. PEI was also used to coat the anodic aluminum oxide (AAO) membrane by cross-flow filtration of the polymer solution through the membranes [27]. AAO–PEI–trypsin preparations with different porosities showed that the enzymatic activity increased with larger pore diameters. In addition, PEI has been used as an enzyme carrier [28], for the protection of oligonucleotides against enzymatic degradation [29], stabilization of electrostatically-immobilized enzymes against washing-off in low pH and/or ionic strength environments [30] and immobilization of enzymes on the surface of electrode [31]. As microcapsules, it is utilized for the extraction of metal [32, 33], trapping of magnetic PEI microcapsules [34-36] and the conversion of ammonia to glutamate by enzymes within lipid-polyamide PEI microcapsules [37]. Our group has successfully immobilized microencapsulated enzymes on the surface of an electrode and investigated the

electrochemical behavior of microencapsulated laccase prepared with a non-conductive polymer (PEI) adsorbed on a glassy carbon electrode [38, 39]. Moreover, PEI microcapsules were incorporated into a paper substance [40] with the goal of developing stable bioactive papers with a high retention of proteins. Other strategies to immobilize biocatalysts on various supports using PEI are discussed in a review article by Bahulekar *et al.*[41]

In spite of apparent advantages of using PEI for enzyme immobilization, many authors reported a decrease of enzymatic activity in PEI-based immobilization systems. For instance, Matella et al [42] studied the immobilized-enzyme system using PEI, glutaraldehyde (GA), and cotton cloth and reported an enzyme inactivation between 50 to 90% with the combination of PEI and GA.

In this thesis, we have compared two simple and effective enzyme microencapsulation methods based on the interfacial polycondensation of PEI and evaluated the microcapsules' properties by several characterization methods. The enzymatic activities of free and encapsulated enzymes were investigated. PEI microcapsules prepared by the emulsion method were strong, intact and with controllable size and size distribution. This method was fast and mild with high productivity and encapsulation efficiency. Capsules prepared by the emulsion method were used for enzyme microencapsulation (Chapter 3 and 4), enzyme paper strip preparation (Chapter 4), bacterial vaginosis (BV) paper strip preparation (Chapter 5) and antibody encapsulation and conjugation (Chapter 6).

## 1.2 Rationale and objectives of the study

The work reported in this thesis is part of Canada's Sentinel Bioactive Paper Network. The goal of Sentinel is to develop technology platforms required for the production of bioactive paper that can detect, capture and deactivate water and airborne pathogens. The overall objective of this study was to make value-added bioactive paper strips that can provide a visible indication of the presence of pathogens or toxic compounds. The specific objectives of the current thesis are:

To fabricate microcapsules with two different technologies and determine the optimal parameters for both methods;

To characterize the produced microcapsules and evaluate their properties, such as the membranes pore size, capsule size and size distribution, wall thickness and wall compositions, all of which are essential factors for future applications;

To compare enzymatic activities and thermal stability, because after microencapsulation, some enzyme properties were modified; to study their new properties, compare the differences and find out the causes;

To fabricate preliminary bioactive paper with enzymes;

To incorporate microcapsules into paper and to examine whether the immobilization step affects the activities of encapsulated active ingredients;

To make bacterial vaginosis (BV) paper that can detect sialidase (neuraminidase), which is related to bacterial vaginosis;

To find a preparation method for antibody microcapsules through microencapsulation or by conjugating antibodies to the outer membranes of microcapsules.

## 1.3 Structure of thesis

This thesis consists of eight chapters. Instrumental and experimental methodologies are explained in Chapter 2. The recent progress in characterization and applications of microcapsules prepared by this technology is reviewed in Chapter 3. Chapter 4 describes the fabrication, characterization of PEI microcapsules and comparison of two microencapsulation methods, in which the optimal parameters of fabrication are studied, and produced microcapsules are characterized. Physically, this microencapsulation method can be classified into two different techniques: the emulsion and the vibration nozzle microencapsulation methods. Changes in enzymatic activities, confirmations and thermal stabilities after microencapsulation were investigated. Chapter 5 is devoted to studying microencapsulated enzymes' properties and discussing the factors that resulted in those changes. Preliminary GOx bioactive paper was fabricated, which can display a color change in the presence of glucose and potassium iodine. A simple, inexpensive and sensitive bioactive paper was fabricated for the self diagnosis of bacterial vaginosis; this is a one-step spot assay. Chapter 6 compares various BV paper strips and highlights the sensitivity and storage stability of this colorimetric biosensor. Moreover, attempts were made on antibody sensitized microcapsules for

the detection and deactivation of pathogens. Chapter 7 examines two types of antibody microcapsule preparation methods: one method is to conjugate antibodies onto the surface of microcapsules and another is to microencapsulate antibodies. Some assays, such as the conjugation method, killing assay and rapid ELISA, will be depicted. Lastly, a brief conclusion and some suggestions for further studies are presented in Chapter 8.

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# Chapter 2 Instrumental and experimental methodologies

## 2.1 Oxygen electrode

The most common technique used in enzyme kinetic characterization is spectrophotometry. Given that the enzymes were encapsulated into microcapsules in this work and its membranes were solid, a traditional spectrophotometer was not suitable for these measurements. Consequently, enzymatic activity measurements were conducted with an oxygen electrode [1-3]. The setup of the oxygen electrode from Rank Brothers is illustrated in Figure 2-1. It comprises a working (Platinum) and reference (silver) electrode. The small (2 mm in diameter) central platinum disc working electrode is the cathode, and $O_2$ diffusing through the semi-permeable Teflon membrane is reduced on this electrode. Set in a well surrounding, this is a silver ring counter acting as a reference electrode. Conduction between the two electrodes is achieved by using a 3 M potassium chloride solution to saturate the paper tissue covering the two electrodes, and then two electrodes were connected by wet tissue paper. On top of this is the key gas-permeable and ion-impermeable Teflon membrane with thickness of 12.7 μm, sealed under a sample solution or suspension in an incubation chamber with a silicone rubber ring. The sample solution is sealed in the incubation chamber.

The oxygen electrode is sensitive to oxygen concentration, whereby the oxygen consumption of enzyme catalyzed reactions can be recorded through it. The scheme

of reaction of encapsulated TvL and GOx is shown in Figure 2-2 and the typical measurement curve is shown in Figure 2-3. We took the slope (oxygen consumption speed) as relative enzymatic activity. Substrate molecules (p-phenylene diamine (PPD) or o-phenylenediamine (OPD) for TvL and glucose for GOx) passed through the membranes of microcapsules and were oxidized; this was catalyzed by encapsulated enzymes.

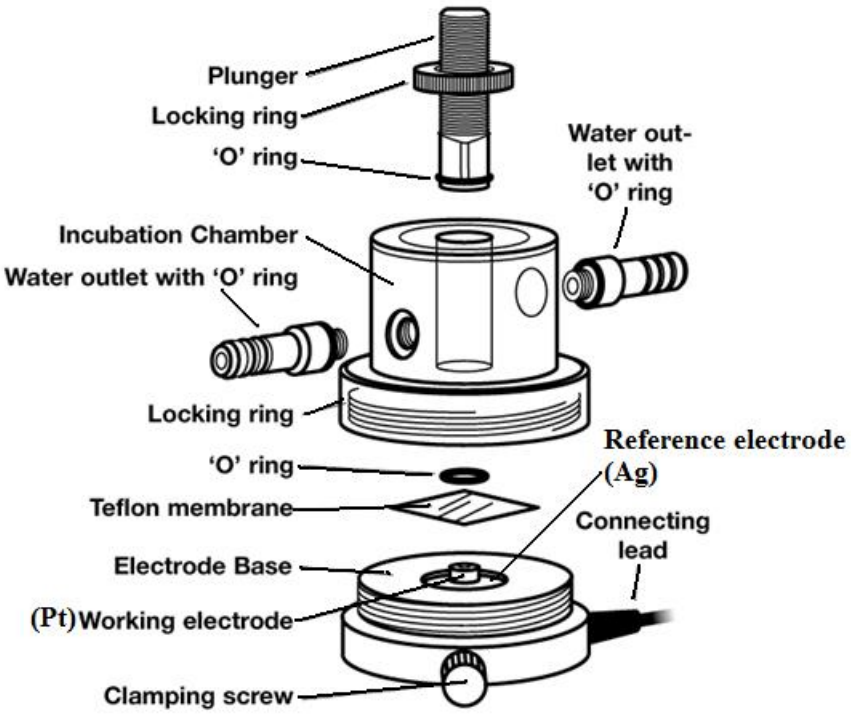


Figure 2- Setup of the Perspex oxygen electrode from Rank Brothers [4].

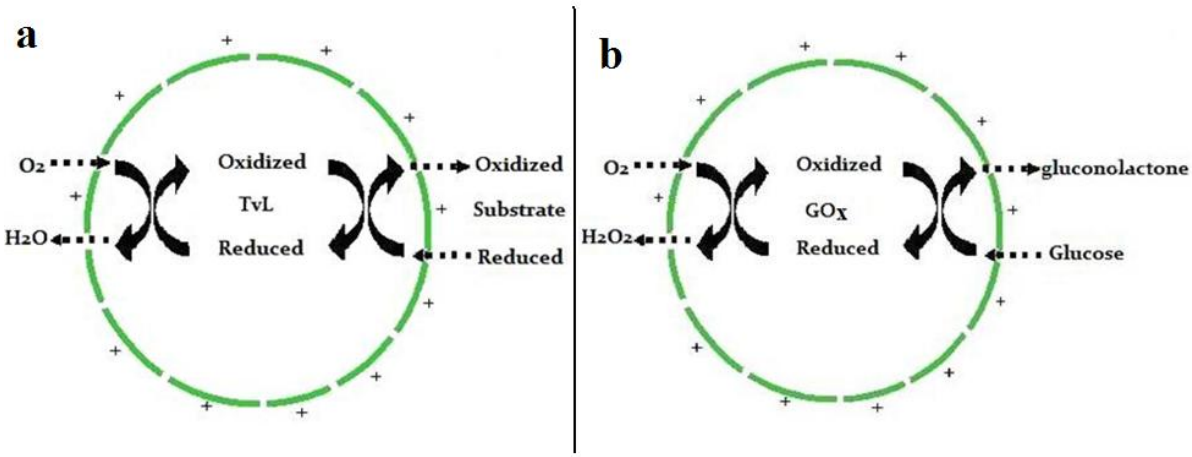


Figure 2- Schematic illustration of the reaction of encapsulated TvL (a) and GOx (b) in oxygen cell.

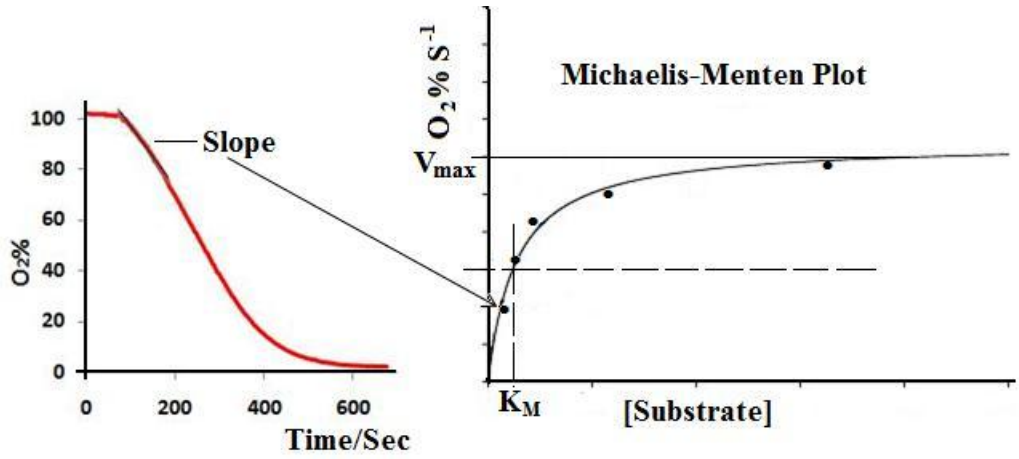


Figure 2- Typical oxygen consumption curve (on the left) measured with the oxygen electrode for a given substrate concentration. The slope of the oxygen consumption curve, representing enzymatic activity, is determined for each substrate concentration and plotted (on the right) to obtain the Michaelis-Menten parameters.

## 2.2 Confocal laser scanning microscope (CLSM) and optical microscope

A confocal laser scanning microscope [5-7] is used for obtaining high resolution images and 3-D reconstruction. In contrast to wide-field microscopy, confocal microscopy is able to produce blur-free images of thick specimens at various depths, and eliminate image degradation of out-of-focus information or glare in specimens whose thickness exceeds the intermediate plane of focus. The principle of the CLSM was developed by Marvin Minsky in 1957 [8]. During the late 1970s and 1980s, advances in computer and laser technology, coupled to new algorithms for digital manipulation of images, led to a growing interest in confocal microscopy [9]. Practical CLSM designs were translated into working instruments by several investigators [10-13].

In CLSM, images are taken point-by-point [14]. Our measurements were conducted on a Leica TCS SP5 II CLSM equipped with three point laser sources: Argon ion (blue and green, 458/488/514 nm), Helium-Neon (red, 633 nm) and Diode-pumped solid-state (DPSS) (yellow, 561 nm), controlled by high-speed acousto-optic tunable filters that allow a very precise regulation of wavelength and excitation intensity. The configuration scheme of Leica TCS SP5 is shown in Figure 2-4.

In the specimen, the light is absorbed by fluorophores, which are artificially added to label molecules. After absorption, fluorophores, for example,

fluorescamine labeled to PEI start to emit light in a random direction and with a wavelength longer than that of the excitation [6]. The objective focuses the laser light on a small spot in the specimen, whereby it collects the fluorescence emitted by the fluorophore within the specimen and relays the images of the specimen onto the detection pinhole. Regular fluorochrome dyes such as fluorescein isothiocyanate (FITC, Maximum Excitation: 490 nm, maximum Emission: 525 nm), sulforhodamine 101 acid chloride (Texas-red, Maximum Excitation: 596 nm, Maximum Emission: 615 nm) and fluorescamine (Maximum excitation: 390 nm, Maximum emission: 465 nm, 475 nm) were used to label poly(ethyleneimine) and enzymes. The 3-D image of half microcapsules prepared by the emulsion method is illustrated in Figure 2-5. The membranes of microcapsules were labeled by Texas-red and the enzyme was labeled by FITC.

## 2.3 Particle size analyzer

The mean size and size distribution of microcapsules were measured by LA-950 laser diffraction analyzer (Horiba). The measurements were made based on their static light scattering. The scheme is shown in Figure 2-6. For particles larger than the wavelength of light, the light scatters from the edge of the particle at an angle which is dependent on the size of the particle. Larger particles scatter light (green line) at relatively smaller angles than light scattered from smaller particles (red line). By observing the intensity of light scattered at different angles, we determined the relative amount of different particle sizes.

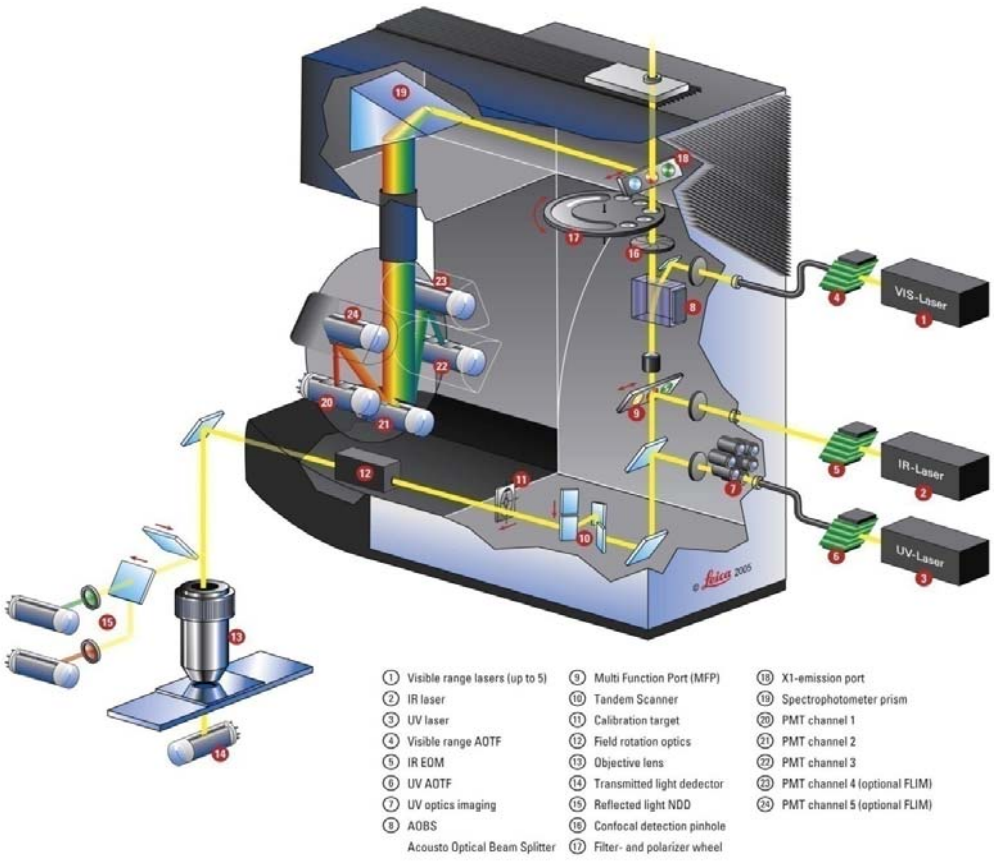


Figure 2- The configuration scheme of Leica TCS SP5 [15]. This system is composed of a regular florescence microscope and a confocal part, including a scan head and laser optics.

The dynamic range of the Horiba LA-950 light scattering particle size analyzer is of 0.01 to 3000 μm, and the diameters of microcapsules prepared by either emulsion or the vibration nozzle method range from a few microns to several

hundred microns (up to 300 μm), which falls within the dynamic range of the instrument. A measurement result for the emulsion method is shown in Figure 2-8b.

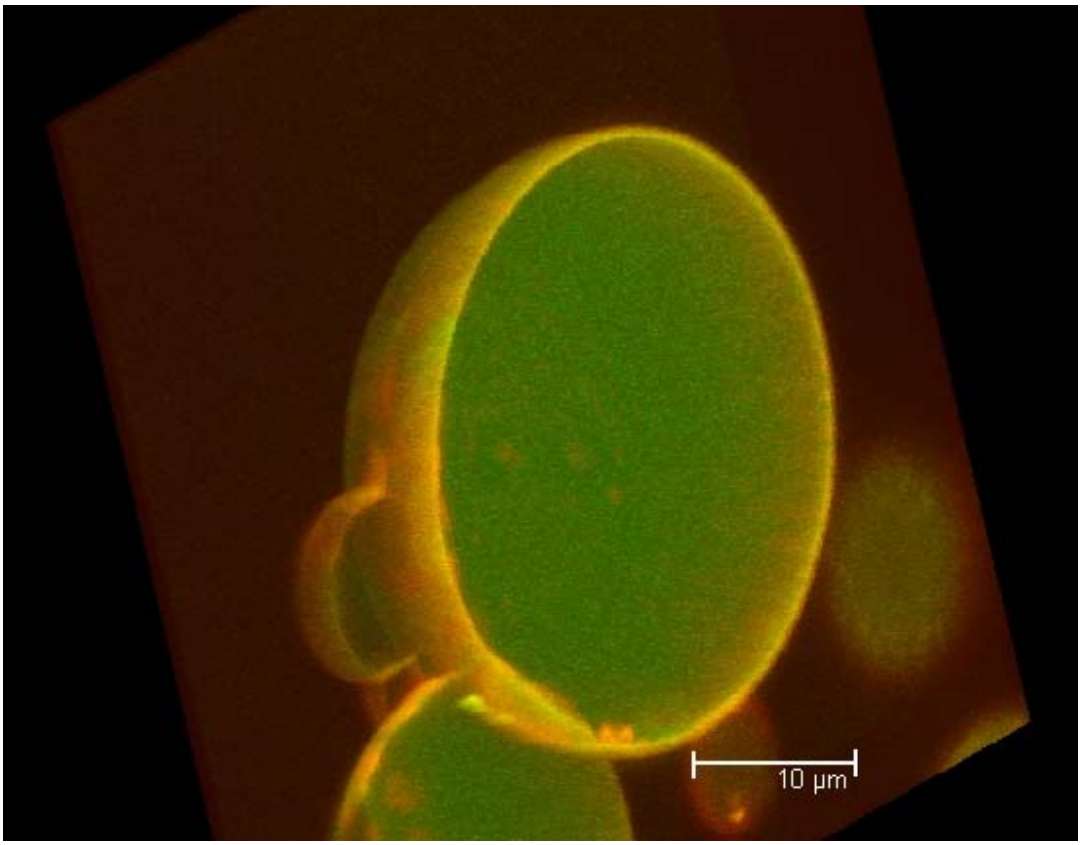


Figure 2- 3-D image of the cross-sections of microcapsules prepared by the emulsion method. Membranes of microcapsules were labeled by Texas-red and core phase was labeled by FITC.

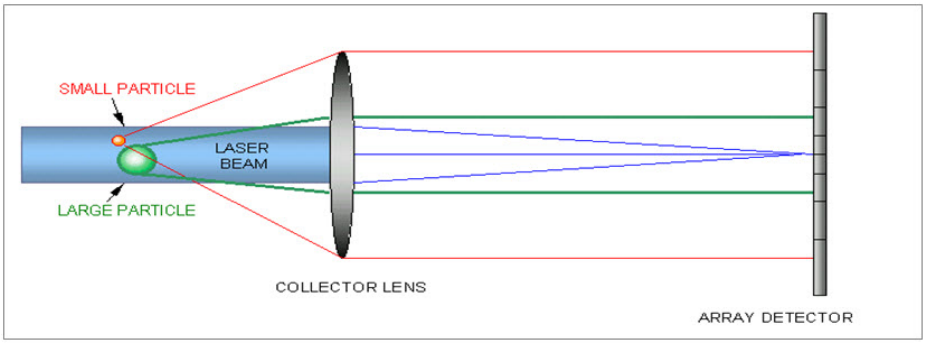


Figure 2- Idealized laser diffraction setup (angle of scatter VS. particle size) [16]. The angle of scatter is inversely proportional to the particle size; the amount of light is proportional to particle size. Large particles scatter more light than small particles.

## 2.4 Emulsion microencapsulation method

Poly(ethyleneimine) polymer was cross-linked by sebacoyl chloride to form the membranes of microcapsules. The external phase consists of the solvent cyclohexane, containing Span 85 (sorbitan trioleate) as an emulsifier to stabilize microdroplets. The core phase is hydrophilic and contains a buffer, PEI and encapsulated components. This technique, first described by D. Poncelet [17] includes two main steps: emulsification and the formation of membranes. The method involves the formation of a polymeric wall around the aqueous phase in order that it is encapsulated. Protein or enzymes are dissolved in emulsion droplets. This kind of microcapsule is obtained from a water-in-oil emulsion by interfacial polycondensation. The polycondensation reaction is rather quick and mild; the first

membrane is formed at the interface between water and oil. In our case, the organic solvent is cyclohexane.

Our group updated this method by modifying some parameters, such as the agitator type, agitation speed, emulsification time etc. The initial emulsification step was crucial to overall process, as it dictated the final size and size distribution of microcapsules. The physicochemical properties of the two phases and the choice of surfactant were undoubtedly the key parameters in determining the equilibrium droplet size. However, only proper association between properties and operation parameters would allow a controllable size distribution [18].

Agitation force and duration of agitation determine the emulsification quality and facilitate the diffusion of the crosslinker from the aqueous solution into the organic phase for the reaction process. A pitched blade turbine (four blades) with a 45° angle offers a good balance between shear and pumping flow; this was adopted for the emulsion method. With sufficient agitation time (longer than 15 min), the mean size of droplets decreased and droplet size distribution narrowed. Microcapsules of small size and size distribution were then generated.

Figure 2-7 shows the schematic illustration of the emulsion method. First, 50 mL of cyclohexane as organic phase containing 0.9 ml sorbitan trioleate (Span 85) was stirred at a certain rotation speed for 2-3 min, and then 10.5 ml of aqueous phase containing pH 8.0 McIlvaine buffer (0.2 M $Na_2HPO_4$ and 0.1 M citric acid),

enzyme (from 1 mg to 50 mg) and 1 ml of poly(ethyleneimine) with a molecular weight of 1,300 Da was added to form water-in-oil microdroplets. After 15 min of agitation, the emulsion was stabilized, droplets remained a constant size after this period. Next 0.5 ml sebacoyl chloride ($C_{10}H_{16}Cl_2O_2$) as a crosslinker in 10 ml cyclohexane was added to react with poly(ethyleneimine) at the interface of all droplets simultaneously and the mixture was agitated for 5 min to mature the membranes. The reaction was stopped by diluting with 50 ml cyclohexane. Supernatant was discarded. Microcapsules were rinsed with 50 ml of cyclohexane to remove any remaining organic residues like hydrolyzed sebacoyl chloride. The microcapsules were then transferred to a Buchner filter funnel and rinsed with Milli-Q water to remove water soluble residues. Small molecules such as hydrolyzed sebacoyl chloride, hydrochloric acid and unreacted poly(ethyleneimine) on the surfaces and inside the microcapsule cores were rinsed off.

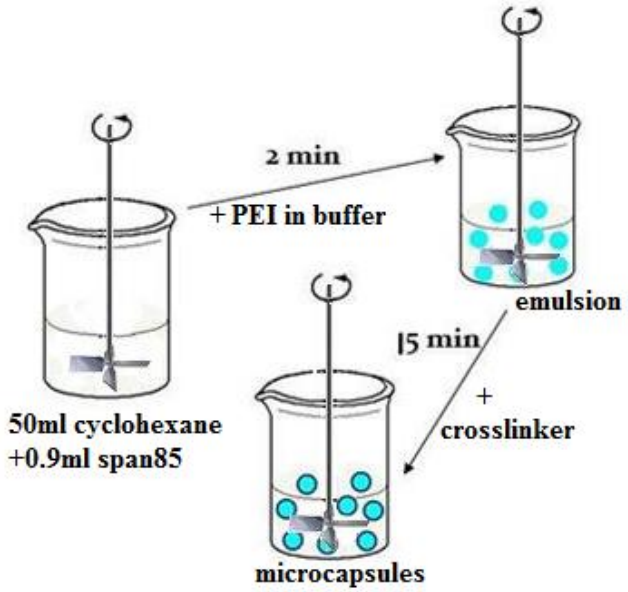


Figure 2- Schematic illustration of the emulsion method.

This method can prepare small microcapsules ranging from a few microns to 50 µm. The properties of produced microcapsules are discussed in Chapter 4. As agitation influences mass transfer in a reactor, this mass transfer can be considered as a crucial parameter for determining the diameter of microcapsules. In general, the diameter of produced microcapsules decreased with increased agitation speed. Microcapsules prepared by the emulsion method have a relatively small size yet a larger size distribution than those prepared by the vibration nozzle method (encapsulator). The size and size distribution of microcapsules prepared by the emulsion method is shown in Figure 2-8.

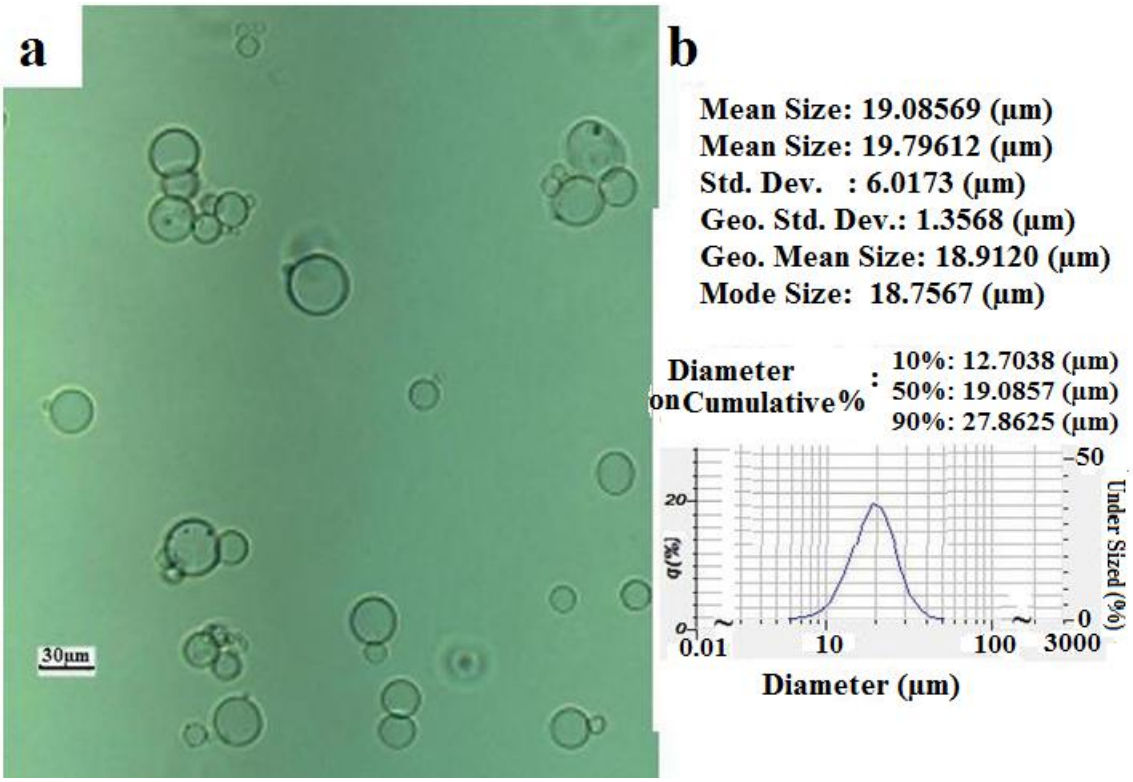


Figure 2- Optical micrograph of microcapsules prepared by the emulsion method with pitched blade turbine (a); size distribution of microcapsules obtained from particle size analyzer (b).

## 2.5 Encapsulator microencapsulation method

The vibration nozzle (or encapsulator) method of microencapsulation is based on the laminar jet break-up technique. The Inotech encapsulator has been used for cell immobilization with different types of alginates. We reported for the first time that PEI microcapsules could likewise be prepared by this encapsulator.

Kondo's [19] US Patent 2 379 817 was issued in 1945 as the initial nozzle-based microencapsulation technology. This program has led to the development of a two-fluid gravity-flow apparatus producing relatively large capsules. Immobilization of living cells or biocatalysts in hydrogels is a well-established technique in a broad, increasing range of different applications. Sodium alginate is the most used polysaccharide as a polymer matrix due to its good biocompatibility and its easy gelling reaction [20-23]. Our work is the first to demonstrate the ability to permanently encapsulate enzymes in PEI microcapsules with an encapsulator. No microencapsulation technique hitherto had permitted the simultaneous production of beads with a narrow size distribution, a high production rate, a satisfactory level of material utilization under mild and nontoxic conditions, under completely sterile conditions and with the ability to scale up [21].

The vibration nozzle technique can be used to produce monodisperse beads of >200 μm in diameter, depending on how the application is scaled up. The break-up of laminar jets is well investigated, but most of the analysis end at the point where the diameter of the neck decreases under a certain value, since mean diameter of microcapsules is about double of nozzle diameter. We tried to break the neck and replaced it with a homemade nozzle with a diameter of 10 μm to produce smaller microcapsules. However, the produced microcapsules were not as small as expected and were lightly aggregated.

This method was performed with a commercial encapsulation device (Inotech IE-50R encapsulator, Figure 2-9 a). The encapsulator was equipped with a 100 μm

nozzle (Figure 2-9 c). Figure 2-9 b shows beads generated from a 500 μm nozzle. Microdroplets were produced by pumping a 10 mL solution containing pH 8.0 McIlvaine buffer and 5% (v/v) PEI with MW of 750 kDa (PEI 750) through the nozzle while applying vibrations at a given frequency. Formed microdroplets fell into a hardening solution containing 50 mL of cyclohexane, 0.7 mL of span 85 and 0.4 mL of sebacoyl chloride, which was agitated by a magnetic stir bar. The scheme of the vibration nozzle method is shown in Figure 2-9 d.

The homemade nozzle is a glass needle with a nozzle diameter of 10 μm. In general, the bead diameter of a Newtonian liquid is about twice the diameter of the nozzle. However capsules we obtained from the nozzle of 10 μm were much bigger than 20 μm and large microcapsules were attached to several small microcapsules (Figure 2-10). As shown in Figure 2-10, microcapsules aggregated and had a large size distribution.

## 2.6 Scheme of enzyme microcapsules

The fabrication process for enzyme microcapsules is similar to that of empty microcapsules. The only difference is the enzyme-containing aqueous phase with PEI. Given that enzyme molecules contain primary amine groups that can react with the crosslinker, enzymes after the microencapsulation process can be found embedded both in the membranes and within the core (Figure 2-11). A low molecular weight of PEI (1.3 kDa) was used for the emulsion method, yet only a high molecular weight of PEI (750 kDa) can produce strong, intact and spherical

microcapsules using the vibration nozzle method. According to the pore size measurement described in Chapter 4, the membranes of microcapsules were semi-permeable and allowed diffusion of small molecules such as PEI with a molecular weight of 1.3 kDa (PEI1.3), but not large molecules such as laccase (67 kDa) and PEI with a molecular weight of 750 kDa (PEI 750). Microcapsules prepared by the emulsion method only contained enzymes and water molecules after rinsing with Milli-Q water since PEI 1.3 molecules were washed off (Figure 2-11 Emulsion method). However, large microcapsules prepared with high molecular weight PEI by the vibration nozzle method contained PEI 750 as well as enzyme and water molecules; only the very small molecules such as hydrochloric acid and hydrolyzed sebacoyl chloride could pass through and be rinsed away (Figure 2-11 Vibration nozzle method).

PEI interacts with laccase, and its presence in the core phase decreased the enzymatic activity of laccase, which is discussed in Chapter 4. The toxicity of PEI increased with increased molecular weight [24, 25], suggesting that PEI 750 is more toxic than PEI 1.3. In addition, the yield of the emulsion method is about twice that of the vibration nozzle method. In comparison, the emulsion was deemed more suitable as an enzyme encapsulation method for our biosensor platform. Given that the size of microcapsules influences the immobilization quality, microcapsules prepared by the vibration nozzle method were too big to be immobilized into paper pulp.

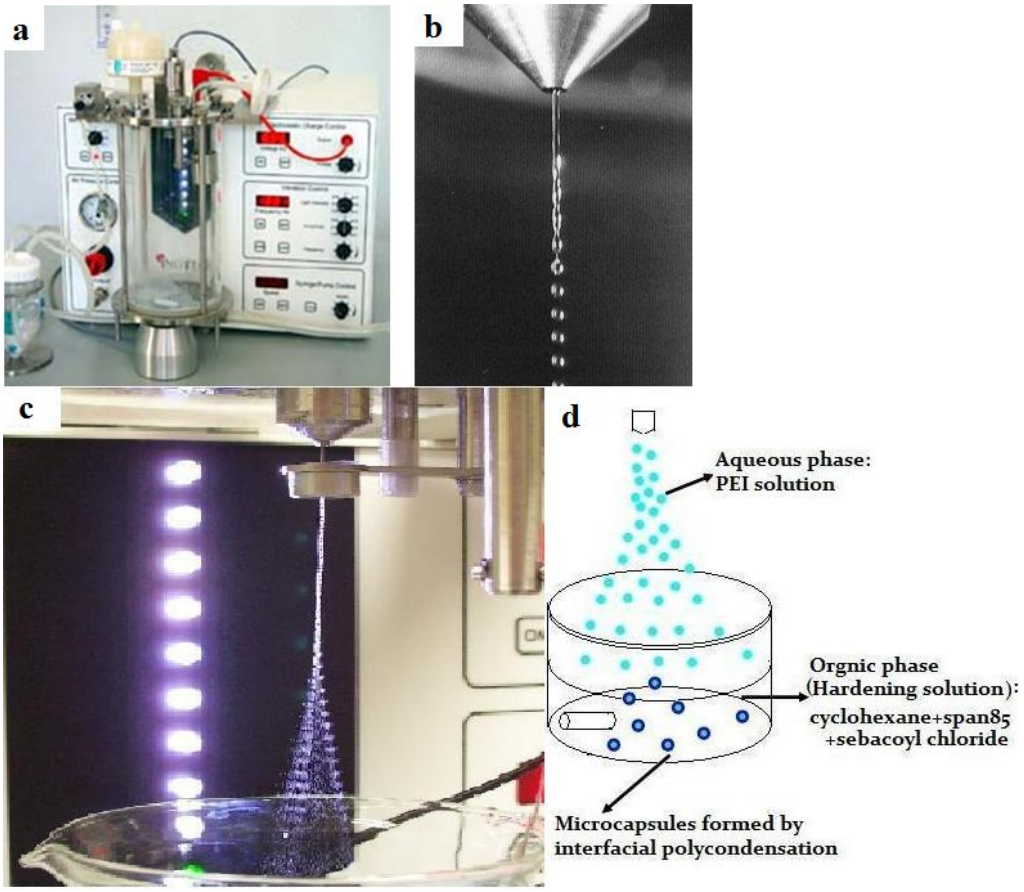


Figure 2- Scheme of the encapsulator and vibration nozzle method. Inotech IE-50R encapsulator (a); Beads generated from 500 µm nozzle (b); Microdroplets generated from 100 µm nozzle at optimal condition (c); Scheme of PEI microcapsules formation by vibration nozzle method (d).

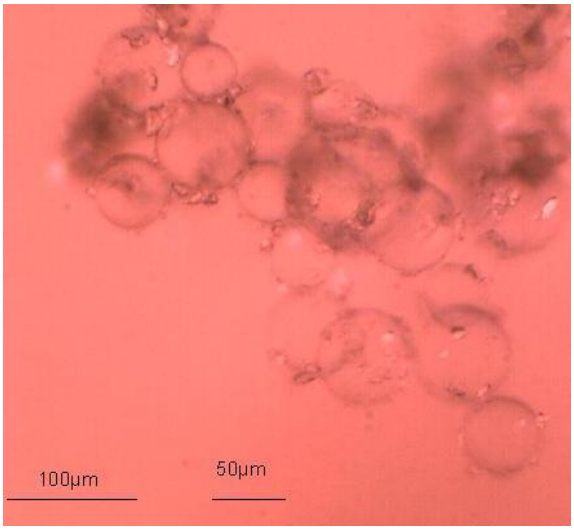


Figure 2- Optical micrograph of microcapsules prepared by an encapsulator equipped with a homemade nozzle with a diameter of 10 µm.

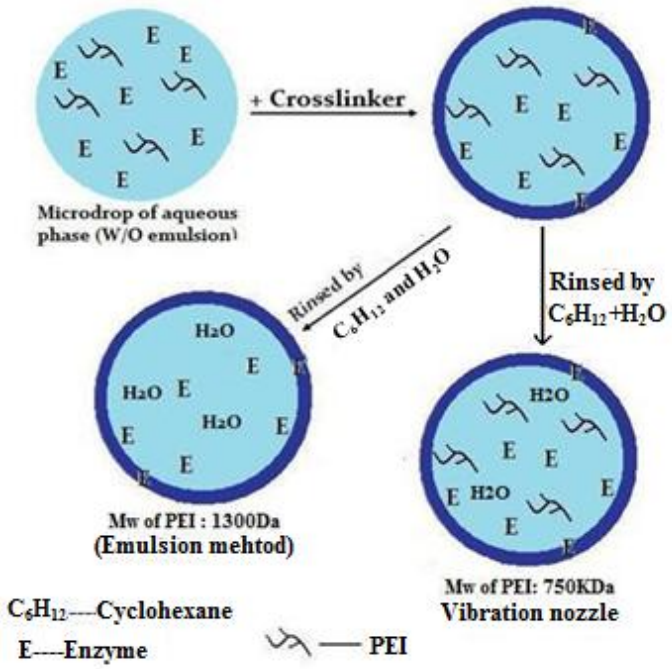


Figure 2- Scheme of the enzyme microcapsules formation.

## 2.7 Preparation of paper strips

In the universal paper-making method, a dilute suspension of wood pulp in water is drained through a screen and the interwoven pulp is laid down on the screen. Suspended water is removed from the sheet by pressing and drying in order to make paper. The paper handsheets were prepared in our lab using simple equipments. Hard wood paper pulp from Paprican was used as substrate for the preparation of handsheets, including empty paper (without microcapsules, as negative control), enzyme paper (containing enzyme microcapsules), BV paper (used in BV test, containing microcapsules soaked in BCIN and NBT solution) and microcapsule paper (containing empty microcapsules).

Paper pulp was suspended in water or buffer depending on the experiment, and then agitated to form a homogeneous suspension before transferring it to a Buchner filtration funnel for quick drainage. Similarly, empty microcapsules or enzyme microcapsules suspension in water or buffer were mixed with a homogeneous pulp suspension, gently agitated, and then transferred to a Buchner filtration funnel for drainage. Wet paper was dried by purging with $N_2$. Dried paper handsheets were cut and kept in separated tubes, which were later used for the colorimetric test and storage stability test. These paper strips are only for single use and can facilitate detection purposes by signaling a color change. Figure 2-12 shows hard wood paper pulp before paper-making (a) and papers with or without microcapsules labeled by fluorescamine (b, c), in which microcapsules were labeled by fluorescamine;

emitting green color under ultraviolet excitation (Figure 2-12 c right hand paper). This paper contained microcapsule aggregates, suggesting that capsules were not uniformly dispersed in paper pulp before filtration. Generally speaking, with this technique it is possible to achieve a better dispersion yet not to obtain monodispersed microcapsules. Thus this kind of colorimetric paper strip generates uneven intensity, which in practice improves visual ability.

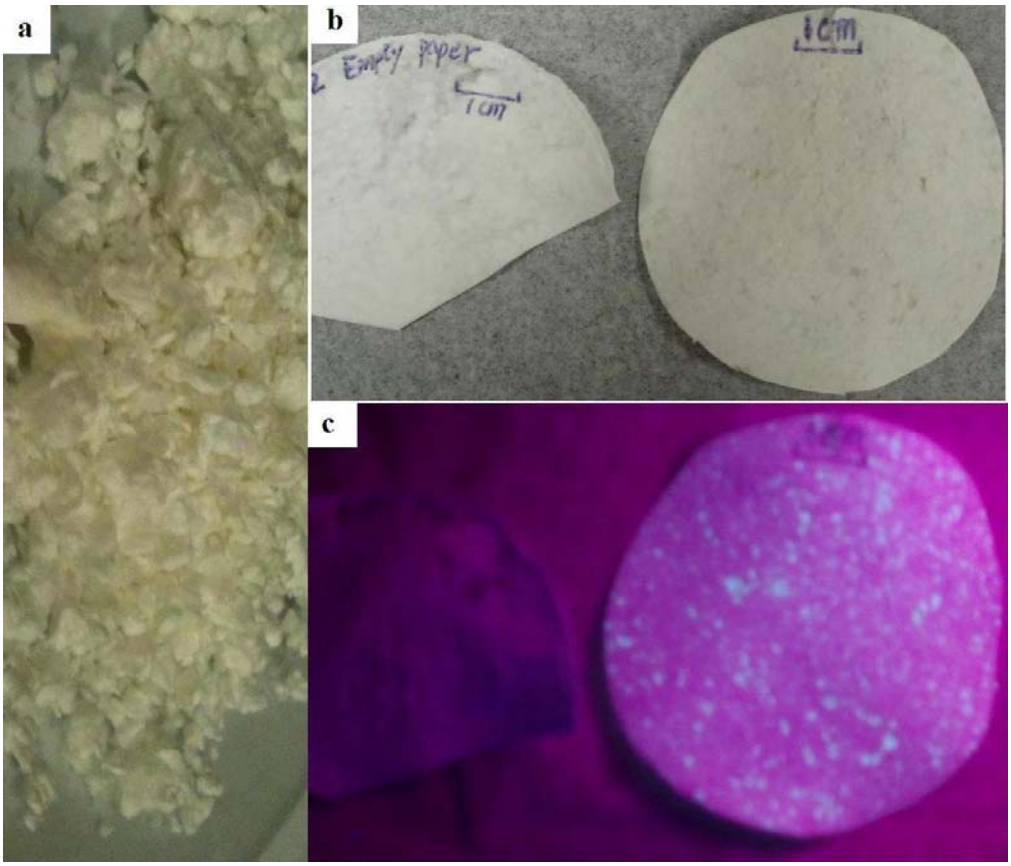


Figure 2- Dry bleached hard wood pulp from Paprican (a) and papers with microcapsules labeled by fluorescamine (right side in b and c) and without microcapsules (left in b and c).

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# Chapter 3 Characterization and applications of microcapsules obtained by interfacial polycondensation[1]

## 3.1 Abstract

This review highlights the materials, mechanisms and applications of microencapsulation by interfacial polycondensation in different areas. This technology entraps active ingredients inside microcapsules/microspheres, having an average diameter ranging from nano size to several hundred microns. Polycondensation reactions take place at the boundary of two phases to form the shells of microcapsules or matrix microspheres. The emulsions can be classified into three types: water-in-oil, oil-in-water, and oil-in-oil. According to the hydrophilic-lipophilic property of core phase, different active substances, such as proteins, enzymes, insecticides, herbicides, vitamins, catalysts, drugs, essential oils, dyes, and phase change materials have been successfully incorporated into different microcapsules/microspheres. Based on the shell-forming materials, this technology is capable of preparing polyamine, polyurea, polyurethane, polythiourea, polyester, polyepoxide, polyacrylamide, and polysiloxane microcapsules. Over the past two decades microcapsules prepared by interfacial polycondensation have been widely used in

[1] This chapter was published as a review article in Journal of Microencapsulation.

Yufen Zhang, Dominic Rochefort, Characterization and applications of microcapsules obtained by interfacial polycondensation, Journal of Microencapsulation, 2012**,** 29(7), 636-649**.**

carbonless paper, cosmetics, pharmacy, agriculture, energy storage/transfer, thermal insulation/regulation, and information and magnetic recording.

**Keywords**

Characterization, Polymers, Permeability, Interfacial polycondensation, Agrochemicals

## 3.2 **Introduction**

Interfacial polycondensation is founded on the classic Schotten-Baumann reaction between an acid chloride and a compound containing an active hydrogen atom [1, 2]. Its mathematical model was described by Berezkin [3]. In this paper, microencapsulation by interfacial polycondensation includes all reactions where reactants meet at the interface of an emulsion and react rapidly.

During the past two decades, interfacial polycondensation has found increased applications due to its high yield, moderate and fast syntheses, and controllable release characteristics, which in turn is a straightforward and frequently applied method of producing microcapsules. The encapsulation of core substances by interfacial polycondensation has been widely used in different fields. However, with the exception of one critical review in French published in 2007 [4], there has been no recent review of the main reference works on the subject of interfacial polycondensation since Morgan [5] and Arshady [6] back in the 1980's. Since then, many new types of microcapsules were synthesized for various applications by different polymers. The present survey provides a new general review of various interfacial polycondensation procedures employed for the

preparation of microcapsules and microspheres. It outlines the new features of characterization and novel materials of shell-forming, and discusses the recent progress in different applications.

Interfacial polycondensation is a traditional and well-understood microencapsulation technology, and has become increasingly popular in energy storage/transfer industries as well as in many other applications such as pharmaceutics, agriculture and cosmetics. Future studies of microencapsulation by interfacial polycondensation will focus on areas, such as encapsulation efficiency, activity of entrapped materials, storage stability, functionality and cost.

## 3.3 **Classification, mechanism and characterization**

### 3.3.1 Classification

Microcapsules/microspheres can be classified in terms of their morphology and microemulsion process.

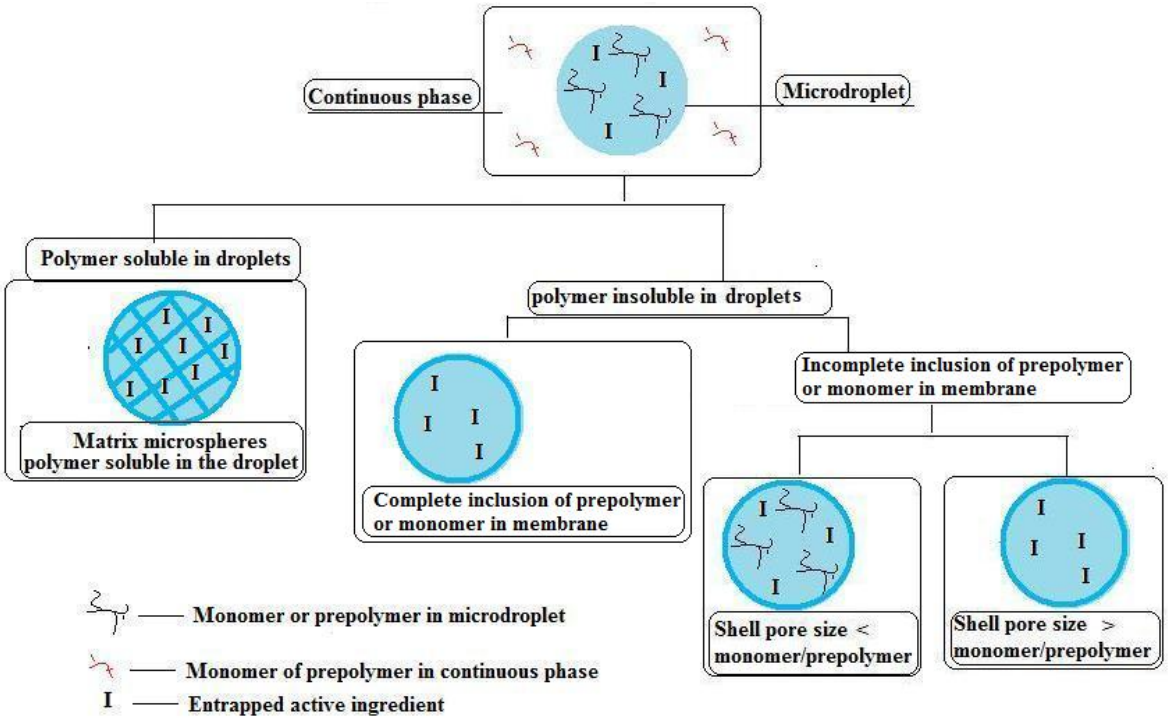


Figure 3- Scheme of microcapsule formation by interfacial polycondensation.

Based on their morphology, microcapsules/microspheres can be classified into hollow microcapsules [7-9], porous microspheres [10] and matrix microspheres [10, 11].

The preparation of microcapsules by interfacial polycondensation involves the emulsification process. In terms of the solubility of emulsion droplet as soft template, the emulsion can be classified into three types: oil-in-oil [12], oil-in-water [13-15] and water-in-oil [16-18].

### 3.3.2 Mechanism

The basic principles of interfacial polycondensation are explained in previous reviews, books, and patents [5, 6, 19-25]. In short, monomers or prepolymers contained in two immiscible phases are cross-linked to form microcapsules/microspheres. Figure 3-1 represents the scheme of microcapsules/microspheres formation. If both the polymer and crosslinking agent are soluble in the droplets, microspheres are formed; otherwise, the membranes are formed around the microdroplets, namely microcapsules [6]. Interfacial polycondensation is a two-step process: the formation of stable microemulsion and polycondensation reaction at the interface of an emulsion.

The performances of microcapsules are largely dictated by the physicochemical properties of the materials, thus the microencapsulation system should be selected in terms of end applications. Microcapsules with different shell materials and active ingredients were investigated for various applications, which are listed in Table 3-1.

Table 3- The core and shell phases and applications of microcapsules by interfacial polycondensation.

| Shell materials | Core materials | Diameter /μm | Applications | References |
|---|---|---|---|---|
| Polyamine: HMDA, TDC | Essential oils, Poly(acrylic acid) gel, oil | 210, 0.5-20 | Cosmetics (essential oils), Extraction of metal cations | [13, 14, 26-30] |
| Polyamine: PEI, TDC | Enzymes in hemoglobin | 100 | Pharmacy | [31] |
| Polyamine: SMA, polyamines | Insect sex pheromones | 50-60 | Insecticide | [10] |
| Polyamine capsules-$Co(NO_3)_2•_6H_2O$ | Polyacrylic acid | 4-12 | Catalysis, magnetic recording | [32] |
| Polyamine /Polyurea | Hemolysate | <1-63 | Promising blood substitute | [33, 34] |
| Polyamine: PEI, SC, TC, HAD | Empty, Alginic acid | 313-865 | New microfluidic device, metal extraction | [35, 36] |
| Polyamide-Polystyrene | Empty | 11-83 | Morphology study | [15] |
| PU: IPDI, propanetriol | Organic liquids | 0.17-0.59 | Nanoreactor | [37] |
| PU and poly(ether urethane) | Essential oil, α-tocopherol, MCR | 0.15-0.5 | Drug carrier, cosmetics, pesticide | [38-41] |
| Poly(ortho ester); polyester | DHEA, BSA, agrochemicals | 1-500 | Controlled release, agriculture | [12, 42, 43] |
| Polyepoxide: EGDE, HMDA/DETA) | VB6 and magnetite | 10-100 | PTFE membrane device | [16, 44, 45] |
| Polysiloxane: OES, TEOHS | Empty | <1-several | Kinetics study | [9] |
| Polythiourea: TDITC, OPD | Carbofuran | 3 | Pharmaceutical | [46] |
| (Photo-crossinking) Polyurea: polymeric isocyanate with a polyamine | TCMP; Photo-initiators; / benzalkonium chloride; limonene oil | 1-125 | Agriculture, Pharmacy, information recording | [47-50] |
| Polyurea: MDI, HMDA, HMDI, DETA; Polyamine | CDEMA | 3-10 | Kinetic study, Agriculture: Herbicides | [51-57] |

| | | | | |
|---|---|---|---|---|
| PU/polyurea: MDI, HPVA. DEG and TMP | Dyes | 0.5-30 | Cosmetics (lipsticks) | [41, 58] |
| Polyurea: TDI, amines | Reactive amines; Chlorpyrifos, DBDPO | 4-36 | Self-healing, agriculture, controlled release, improve thermal stability | [18, 59, 60] |
| Polyurea, polythiourea and PU | Hydrophilic compounds (formamide) | < 1 | Silver nanoparticles | [8] |
| TDI with EDA (Polyurea) / DETA (PU);UF; Nylon-6, 10: HDA, SC ; PAPI; UG | Phase change materials, BSA, ink, perfume, detergent | 1-300 | Energy storage/heat transfer, thermal regulation, clinics, carbonless paper, perfume and laundry | [3, 61-74] |
| Alginate/polyacrylamide | dibutyl sebacate | 500-1400 | Bioremediation of atrazine | [75, 76] |
| HSA/TPC, AC/POPA | empty | 10-40, 96-249 | Morphology and microencapsulation process study | [77, 78] |

### 3.3.3 Characterization

*Size and size distribution*

The measurement of size and size distribution of microcapsules usually conducted on the optical microscope, confocal laser scanning microscope (CLSM), scanning electron microscope (SEM) and particle size analyzer.

Landfester [79] reviewed the structures in miniemulsions, typically in which the mean diameters of microcapsules/microspheres by interfacial polycondensation are influenced by the type of blade, agitation speed and emulsification time [64, 80-83]. Given such influences, the mean particle size increased with decreasing agitation speed. The stirrers

used were selected according to the properties of the solution, such as viscosity. High agitation speed can provide a high break up force to generate small microemulsions. The emulsification time should be long enough to reach an equilibrium between coalescence and break up forces, leading to a stable droplet size [84], In other parameters such as viscosity, the concentration of emulsifier and molecular weight of the core phase can likewise influence the mean diameter of microcapsules. Viscosity of the stock solution was also critical to capsule formation [85, 86]. For instance, compared to the process of urea-formaldehyde microcapsules containing dicyclopentadiene with a less viscous solution [80], microcapsules made by McIlroy [18] are several times larger at a rotation speed of 1500 rpm yet nonetheless exhibit a similar size to previous capsules prepared at higher speeds; this is likely due to a shear thinning of the continuous phase. Furthermore, it was found that the mean particle size increased with increasing the content and molecular weight (eicosane > octadecane > hexadecane) of phase change materials at the same stirring rate. In addition, the mean particle size decreased with increasing the content of the emulsifier [64]. Generally, an emulsifier with lower interfacial tension produces smaller microcapsules [87]. Ni et al. [88] found that the diameter of microcapsules decreased as the emulsifying time was increased at the initial three minutes, beyond which no appreciable changes in the diameter were observed. The increase of content and Mw of core materials and the decrease of emulsifier concentration can increase the viscosity of the emulsion, thus these changes can lead to similar results. Generally, emulsification is the driven process for size and size distribution of microcapsules/microspheres. In that the two-phase fluid has to be homogenized, the stirrer must provide a sufficient pumping effect and shear stress to

obtain an adequate droplet breakup for the final optimal size. Study on polyurethane microcapsules by Frere et al. [89] shows that diameter of microcapsules increases as temperature is increased, but mechanical resistance decreases with increase of diameter. It was observed that different types of surfactants and variation in pH and ionic strength of the continuous phase affected the diameter of the miniemulsion droplets and the resulting microcapsules [37].

As for microcapsules prepared with other instruments, the factors influencing the size varied with different instruments. Where microcapsules were prepared with PTFE membranes such as water-in-oil and oil-in-water emulsification devices, the PTFE membrane nominal pore size and permeation pressure of dispersed phase can affect the size and size distribution of produced microcapsules [16, 44]. With regard to microcapsules prepared with a commercial encapsulation device such as an Inotech Encapsulator, the agitation speed doesn't affect the size of formed microcapsules; the nozzle diameter and pump speed of aqueous phase are key factors for the size distribution [17, 90]. This technique is based on a laminar jet breakup technique and is capable to produce mono-dispersed microcapsules at large quantities. Multi-fluid passing through the nozzle was broken up by applying a vibration at high frequency. Microdroplets were formed before reaching contact with the stirred hardening solution. Therefore, the function of agitation is to disperse produced microcapsules. Similarly, Quevedo [35] found that the size of the capsule produced by the tubular microfluidic device gradually decreased with increased aqueous flow rate and, hence, with increasing Reynolds number. Alternatively, the disperse phase flow rate may be slowed in order to decrease the capsule size. Accordingly, the

factors that influence the mean diameters differ from different polycondensation systems. Mahabadi et al. [91] found that the net effect of two kinetic processes shows an optimal shearing time where the smallest particle size can be attained, and this optimal time is a function of several primary variables. The depletion of surfactant/stabilizer, especially when surfactant concentration was low, resulted in rapid growth of particle size, eventually suspension failure.

*Wall thickness*

Many requirements such as wall thickness are strongly dependent on the application of the capsules. For this reason, studies regarding to what extent, and by which means the thickness of the capsules wall can be controlled are of considerable interest. The wall thickness varies from a few nanometers to tens of microns, which is controllable by changing some parameters, such as the nature and composition of the two immiscible phases, the concentration of the reactants, physico-chemical properties of the encapsulating agent, the partition coefficient of each reactant between the two phases, chemical nature and concentration of the chain extending agent, type and concentration of the emulsifying agent, the swelling or solubility of the microcapsule wall, and the rate of reaction between the monomers, reaction time, emulsification time, and molecular weight (MW) of the prepolymer. The factors influencing the wall thickness vary for different microencapsulation systems. Study on polyamine microcapsules by Danicher et al. found that wall thickness strongly depends on the reaction conditions: monomers concentration, crosslinking agent fraction and reaction time [83]. Based on the transmission electron

microscope (TEM) micrograph Jabbari [53] found the thickness of the wall does not increase with increased reaction time. Similarly, Poncelet [92] reported that membrane formation was primarily affected by the concentration of crosslinking agents, whereas both poly(ethyleneimine) (PEI) concentration and reaction time appeared to have little effect on the membrane weight. The membrane weight slightly increased with increased PEI concentration and reaction time. Nevertheless, small changes in some parameters can definitely change the wall thickness. For example, the wall thickness of diethylene triamine microcapsules can be controlled from 20-500 nm [10, 53, 93] to 10 μm [94]. Janssen [94] gave the following equation of wall thickness of the capsules during encapsulation. [2]

$$d(t) = d_1 + \sqrt{2D_{2,DETA}C_{0,DETA}(M_w/r\rho_{pol}\beta)t}$$

Measurements of the wall thickness as a function of the reaction time $\sqrt{t}$ for smaller-sized capsules (300-1000 μm) show very good agreement with this equation. Mainly the diffusion rate from microcapsules decreased with increased wall thickness, with the exception of the diethylene triamine microcapsules prepared by Janssen [94]. During the

[2] dt: wall thickness; d1: thickness of top layer of the capsule wall; $D_{2,DETA}$: diffusion coefficient of DETA through sub layer to the capsule wall; $C_{0,DETA}$: concentration of DETA in aqueous phase; Mw: molecular weight of the average polymer unit; r: reaction ratio TDC/DETA; $\rho_{pol}$: density of the polymer; β: volume fraction of polymer in the sub-layber; t: time.

maturation of capsules wall, the pore size increased with increased wall thickness. Addition of diamine to aqueous phase reduces the permeability of the capsules wall and an increase of the ratio of the diamine and the triamine concentration also reduces the permeability [95]. Measurements of the rate of hydrolysis of encapsulated teraphtaloyl dichloride show that no hydrolysis will take place during wall formation, as hydrolysis only takes place in the aqueous phase [96].

*Thermal properties and chemical structures of capsules' shell*

Since shells of microcapsules produced from interfacial polycondensation were composed of new types of materials, which were formed by reactions of minimum two monomers or polymer, their compositions and thermal properties were unknown. Some properties of capsule shells were determined by the following methods. Thermal properties of shells used to be determined by differential scanning calorimetry (DSC) and thermal gravimetric analysis (TGA) [17]. Fusion temperature obtained by DSC measurement can give some chemical structure information, for example, suggesting the presence of defaults in the macromolecular back bone such as irregular bounds of urethane and urea functions [97]. TGA curves can provide differences between capsules-forming material and formed microcapsules in composition as well as the effect of capsules-forming material concentration on capsules composition. Chemical structures were measured by nuclear magnetic resonance (NMR), matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF), Fourier-transform infrared spectroscopy (FTIR) [77, 98, 99] and elemental analysis. The main component throughout the capsules' degradation can be

determined via NMR [100]. MALDI-TOF experiments allowed for the investigation of the microstructure of polymer membranes, thereby proving the presence of some functional groups in the membranes [97]. Quantitative elemental analysis and X-ray photoelectron spectroscopy (XPS) of capsules shells can provide the chemical composition and ratio information of capsule-forming materials that participated in the reaction [17, 36, 101].

*Permeability of shells*

The capsules' wall (or shell) acts as a membrane. Membrane permeability is a crucial parameter for hollow microcapsules and matrix microspheres, since they can affect the reaction between the encapsulated active compound and outer substrate, or influence the release capability of the encapsulated compound. Microencapsulation has two major functions, controlled release and enclosing fragile ingredients with a semi-permeable and biocompatible membrane that would protect them from an attack, for example, the immunoprotection barrier. If membranes of microcapsules have a permeability that is too large, entrapped active ingredients would release rapidly from the microcapsule having the first function, on the other hand, the membranes of microcapsules having the second function can't protect encapsulated ingredients.

Permeability can be detected by fluorescent markers (confocal laser scanning microscope), pore size analyzers or microscopy (TEM, SEM). Pore size and the charge of the shells are the most relevant factors for the permeability. When the shells are net charge, pore size is the key factor. An Autosorb-1 automatic surface area and pore size analyzer can be used to determine the specific surface area and total volume of the microcapsules'

pore. In the vacuum sorption system, ultrapure nitrogen gas and liquid nitrogen gas were used as the adsorbate and coolant. The specific surface area was determined with the intrusion and extrusion curves by the multipoint Brunauer-Emmett-Teller (BET) analysis [29, 102, 103]. The average pore diameter can be determined by the Barrett-Joyner-Halenda (BJH) method [104]. The BJH scheme may be summarized in the following formula:

$$v_{ads}(x_k) = \sum_{i=1}^{k} \Delta V_i\left(r_i \le r_c(x_k)\right) + \sum_{i=k+1}^{n} \Delta S_i t_i(r_i > r_c(x_k))$$

In this formula, $v_{ads}(x_k)$ is the volume of (liquid) adsorbate c [$cm^3/g$] at relative pressure $x_k$ (calculated from the value of adsorption expressed in [$cm^3/g$ STP] by $v_{ads}(x) = 0.0015468\ a(x)$ ), pore volume V is given in [$cm^3/g$], S is the surface area [$m^2/g$] and t is the thickness of the adsorbed layer (in appropriate units).[3]

The straightforward method for pore size measurement is to cut the microcapsules and observe the cross section cuts by TEM or SEM according to the pore size [26, 105, 106]. The obtained photos are shown in Figure 3-2. The membrane cut-off also can be performed on the CLSM by using different Mw of fluorescent markers [17, 107, 108]. The pore size can be controlled by changing some experimental parameters, such as the ratio of

[3] x: relative pressure, $x=p/p_s$, $p_s$ is saturation pressure; a(x) : adsorbed amount (cc/g at STP), STP: standard temperature and pressure (0°C and 0.986 atm)

crosslinking monomer/prepolymers, molecular weight of prepolymers, reaction time, concentration of chain-extending agent, and maturation time. The charge property of the microcapsule's membrane is another important factor in the permeability. The capsules will show charge-controlled permeability when the shells are charged. For example, PEI-SC microcapsules having different charge properties at various pHs showed various permeability. Shells are positively charged at pH 5. Typical laccase anionic mediators like $ABTS^{2-}$ were found to accumulate in the capsule wall and prevented an efficient electron transfer with the electrode. PPD is a monovalent cation around pH 5 and is able to permeate through the membrane [109]. However, at a higher pH, net charged shells allowed the diffusion of fluorescein isothiocyanate labelled poly(ethyleneimine) (PEI-FITC) [17]. Similar results were found for the multilayer microcapsules [110]. The membranes of microcapsules have highly sensitive permeability tuned by probe charge and environmentally controlled gating. They can completely reject negatively-charged probes, yet attract positively-charged species to form a higher concentration in the capsule interior. Montasser et al. [34] found that the permeability of polymeric wall of polyamine/polyurea microcapsule is related to its crystallinity and contributes to the growth of nanocapsule membrane by the diffusion of the hydrophilic monomers to get stable colloidal suspensions.

*Morphology*

Surface morphology is an essential character of microcapsules, which can provide useful surface feature information such as thickness, porosity and smoothness for the final

application, and effect different reagents and parameters on the formation and dispersion of microcapsules. Surface morphology of shells used to be conducted on different microscopes, such as optical microscope [99, 102], AFM [109, 111, 112], SEM [30, 57, 99], TEM [53, 98] and CLSM [102, 113]. Most membranes have a porous structure which is controllable by adjusting some parameters. Morphological properties of membranes are frequently sensitive to the nature of monomers and solvents [114]. Essawy [15] found that an indispensable aspect controlling the whole process at almost any stage and condition is the composition of the phases and the distribution as well as the state of all ingredients (not merely the reactants) between the phases. Figure 3-3 is a typical image of a membrane obtained from SEM [15]. Morphology of the capsules membrane in dependence on the amount of shell components while keeping the molar ratio between the TPC and the amine components (1,6-hexamethylenediamine + diethylenetriamine)) unchanged (1:2). The internal structure of the microcapsules was investigated with TEM by Jabbari [53]. The TEM micrograph in Figure 3-4 shows the cross-section of microcapsules at 14 000 magnification. According to this image, the microcapsules have a micro-reservoir structure in which the wall extends well into the core and the active agent is accommodated by the micro-reservoirs, distributed uniformly throughout the entire volume of a microcapsule.

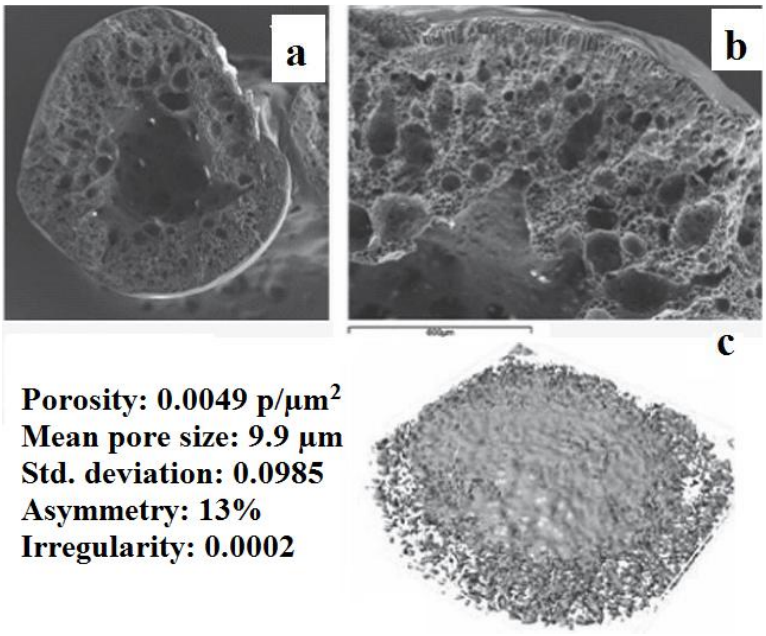


Figure 3- (a) and (b) SEM cross-section micrographs and IFME® numerical results of (b). (c) 3D reconstruction of the 90 µm thickness central part of the capsule from Confocal results (without cutting the sample). Reprinted from publication [106], with permission from Elsevier.

## 3.4 **Applications**

Microcapsules prepared by interfacial polycondensation provide permanent and cross-linked membranes, such as polyamine, polyurea, polyester, polysiloxane, polyurethane and polyepoxide, allowing the entrapment of different active ingredients. This has been used in various fields including the paper industry, agriculture, pharmacology, dye, (solar, heat, nuclear) energy storage/transfer, thermal regulation/insulation, and information and magnetic recording. These are listed in Table 3-1.

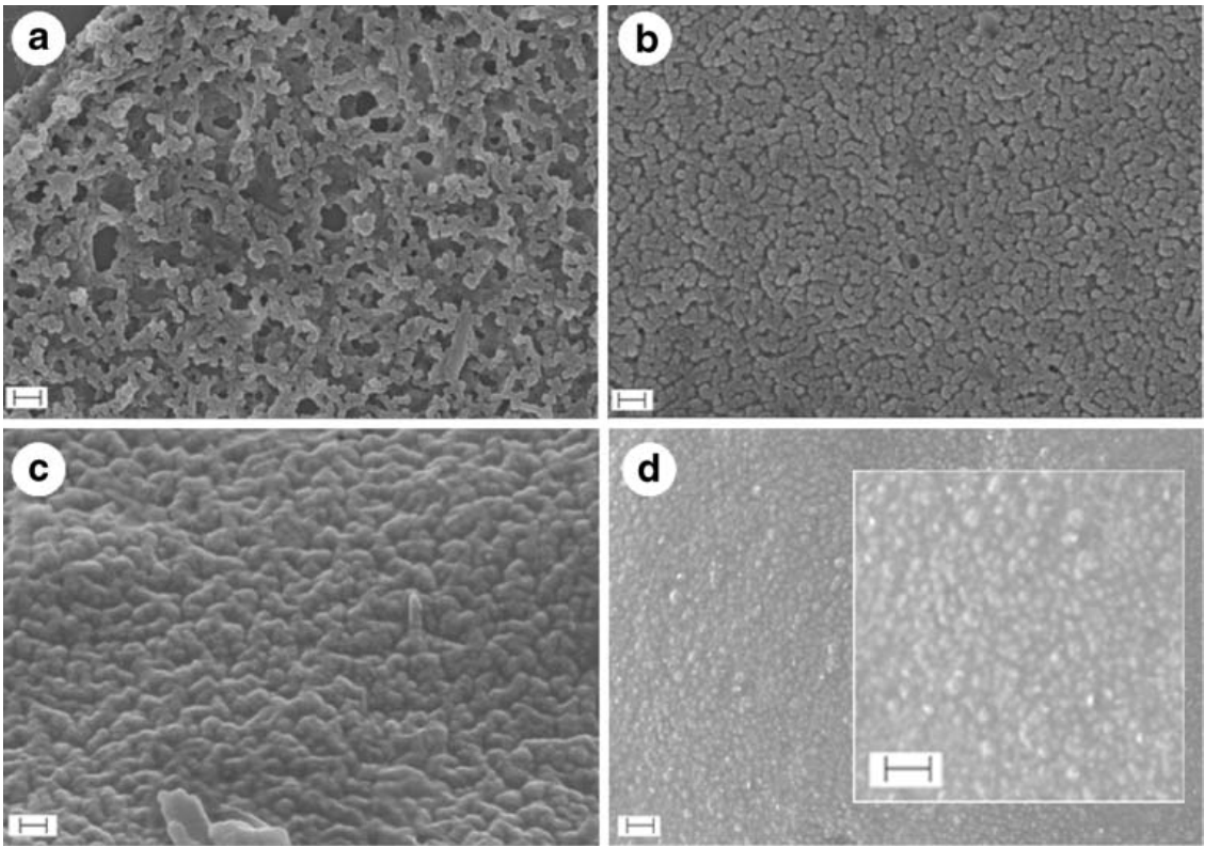


Figure 3- Morphology of the capsules membrane in dependence on the amount of shell components while keeping the molar ratio between the acid dichloride (TPC) and the amine components (HMDA+DETA) unchanged (1:2); the amount of shell components varies in the order of TPC/amine 0.5/1 (a), 1/2 (b), 2/4(c), 3/6 (d); the bar corresponds to 200 nm (a, b, d) and 100 nm (c). Reprinted from publication [15], with permission from the authors.

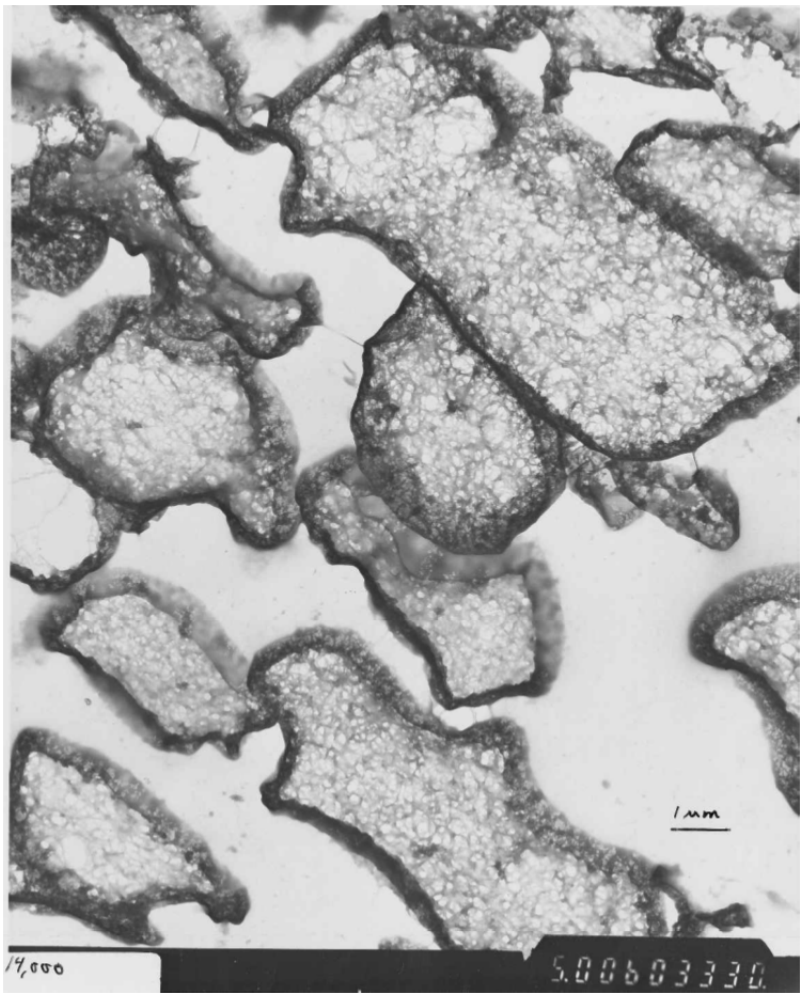


Figure 3- TEM micrograph of the cross-section of microcapsules at ×14 000 magnification. The shadows around the boundaries of the microcapsules are due to folding of the wall at the edges. Thickness of the polymeric film surrounding the microcapsules and the polymeric films forming the internal structure of the microcapsules are in the order of 25±50 nm, respectively. Reproduced with permission of Informa Healthcare [53].

### 3.4.1 Microencapsulation of phase change material (PCM)

Microencapsulated PCM work, as unique thermal energy storage units, have been studied by many researchers and developed by companies, such as BASF Corporation for building applications and Microtek Laboratories, Inc. for increased energy efficiency and thermal regulation.

Since the 1980s, microencapsulated PCM technique has been widely developed. This can enlarge the utility fields of the PCMs, since microencapsulation may protect PCMs from reactions to the outside environment. In addition, it can supply the larger heat transfer area and controllable changes in storage material volume as the phase change occurs, mask highly toxic PCM, make it is easier to handle in practical applications, and allow portable energy storage systems. PCMs have the ability to change their state at a certain temperature range, and can store and release large amounts of energy during the phase changing process [115-117]. Therefore, the benefit of microencapsulated PCMs extend their application use in various areas including thermal-regulated fibres, fabrics, coatings and foams [64, 69, 118-121], thermal insulation [118], heat/thermal energy storage, heat transfer and thermal regulation [61-63, 66, 68, 70, 71, 122], solar energy utilization [69, 117, 120] , and building materials [120, 121, 123]. For instance, n-octadecane is a well-known PCM providing an unacceptable low thermal conductivity despite its desirable properties. This is considered a major drawback, thus reducing the rates of thermal storage and release during the heating and cooling processes. The encapsulation of n-octadecane can enhance the heat transfer, for this reason it has been extended to the application of outdoor apparel, such as ski wear,

hunting clothing, and smart garments with temperature-regulating microclimates [70, 124]. Nevertheless, this application should be carefully considered due to its high cost. Alternatively, some commercial PCMs, like paraffin waxes for solar heating systems are practical and ideal for many applications since these materials are produced in large quantities and widely used everywhere. In addition, phase change temperatures between -10 and 80°C are possible to manufacture with microcapsules.

The continuous increase in the level of greenhouse gas emissions and the rise in fuel prices are the main driving forces behind efforts to effectively utilise various sources of renewable energy like solar energy. Solar green house is attracting more and more attention [117, 120], which can be classified into two categories: The first is to store and utilise solar energy for different purposes, for example solar cookers, which was thought to have a great potential in hot climate countries. The second is to reflect solar heat that can avoid absorption of solar energy resulting in elevated temperature at the shingle's surroundings, thereby contributing to the so-called heat-island effects and increasing the cooling load to its surroundings or energy consumption needs for air conditioning [120].

### 3.4.2 Agriculture

One of the most important applications of microencapsulated ingredients is in the area of agriculture, such as microencapsulation of fertilizer [125, 126], herbicides [53, 54, 127], pesticides and insecticides [40, 51, 52, 59, 128, 129], insect pheromones and other sex attractants [10, 130, 131] and several other agrochemicals [43, 47, 130, 132]. Microencapsulation could increase the fertilizer, herbicide and pesticide efficiency at

reduced doses due to a controlled release, allowing farmers to apply pesticides and herbicides less often and at low initial concentration and toxicity. The membranes formed by interfacial polycondensation are stable, permitting a more controlled release, thereupon providing the advantage of longer protection to the treated area than microencapsulated fertilizer in polysaccharide shells which can be de-cross-linked in some buffer solution [133].

This is the typical case of drift from the host due to rain or wind when applying insecticides, fertilizer, fungicides and herbicides. Not to mention, volatility is another issue for fertilizer, nonetheless suitable microencapsulation could efficiently solve all these problems.

Polyurea microcapsules [47] were used as carriers for the nitrification inhibitor such as (trichloromethyl)pyridines, which has a great long term stability in the field environment, while maintaining the level of efficacy of non encapsulated inhibitors. This type of microcapsule avoids the disadvantage of fast release described in U.S. Patent 4,746,513 [134], which releases all the nitrapyrin upon contact with moisture, exhibiting the same stability and volatility as the free form. Polycondensation encapsulation has been used to encapsulate agriculturally-active ingredients, particularly to enhance handling safety and storage stability of the active ingredient through the use of polyurethane rather than polyurea microcapsules [135, 136]. The agriculturally-active material by itself is usually insoluble in water, or has an unacceptably low solubility in water, and then it's difficult to be applied by spraying. Therefore, Misselbrook [137] treated herbicides, such as, trifluralin

by microencapsulation to enhance their combinability with water. This process can be used for reducing nitrosamine impurities while microencapsulating agriculturally-active materials containing nitrosamines. Insect pheromones as insecticides are attracting more attention compared to conventional hard pesticides [40, 52]. Sex pheromones could reduce insect populations by disrupting their mating process, and microencapsulation of pheromones could control their release and protect them from oxidation.

### 3.4.3 Pharmaceutics

Therapeutic is one of the most important applications for microencapsulation by interfacial polycondensation. These potential applications include drug carriers for controlled release [38, 39, 46], enzyme artificial cells for clinical trial in hereditary enzyme deficiency diseases and other diseases [31, 138], artificial red blood cells for use in urgent blood loss [33], medicine release, information recording and reflective index adjustable instruments due to the obvious advantages of the photo-crosslinking process. High selective regulation can be conveniently achieved by selecting exposure wavelength and photo-reactive core compounds [48]. Since polymeric nanocapsules from poly(α,β-malic acid)-grafted nano-silica templates are biocompatible, biodegradable and non-toxic to cells and tissues, these nanocapsules conform to green chemistry and are expected to be applied as drug carriers [139].

As early as 1965, Chang successfully encapsulated cells or tissue fragments, and the enclosed materials may be protected from destruction and participation in immunological processes [140]. The recent progress in artificial cells has been discussed in his reviews

[138, 141]. The earliest routine clinical use of artificial cells is in the form of coated activated charcoal for hemoperfusion for use in the removal of drugs, toxins and waste in uraemia and liver failure. Encapsulated cells are being studied for the treatment of diabetes by encapsulation of transplanted islet cells, liver failure, and kidney failure. In addition, the use of encapsulated genetically engineered cells is being investigated for gene therapy [138]. Recent advances of polymeric artificial cells in biotechnology, molecular biology, nanotechnology and polymer chemistry are now unfolding further exciting possibilities in this field [141].

### 3.4.4 Cosmetics

Interfacial polycondensation is one of the classical methods for essential oil encapsulation [13, 14, 27]. Lipsticks [58] and fragrance [142, 143] microencapsulation have been used extensively. US patent 20070065379 showed many different applications in cosmetics. The composition according to the invention can be a composition for caring, making up the face/body or hair composition such as shampoo, conditioner, styling composition or hair dye [144]. Since essential oils are oil soluble, microencapsulation processes are commonly based on the principle of oil-in-water emulsion formulation. Microencapsulation could protect essential oils against degradation caused by environmental factors. Furthermore it can improve their solubility, end efficiency, mask or enhance their taste, turn them into stable compounds and control release [13]. Biatry [145] patented thermally stabilizing microcapsules to protect cosmetic or pharmaceutical composition against the effects of thermal variation.

### 3.4.5 Self-healing

One of the most promising applications of microencapsulation is self-healing. Self-healing composites [146] possess great potential for solving some of the most limiting problems of polymeric structural materials: microcracking and hidden damage. Figure 3-5 illustrates the autonomic healing concept. Healing is accomplished by incorporating a microencapsulated healing agent and a catalytic chemical trigger within an epoxy matrix. Similarly, amine core microcapsules [18] were prepared by interfacial polymerization of an isocyanate and an amine stabilized by an inverse Pickering emulsion, which were isolated, dried, and redispersed in epoxy. Preparation of amine-containing microcapsules enables the creation of self-healing epoxies where the microencapsulated healing agents contain the same chemistry as the bulk matrix. Capsules released the core material upon rupture and were able to cure epoxy to form a polymer film. Although the potential benefits are quite high, the specific composite described here has some practical limitations on crack-healing kinetics and the stability of the catalyst to environmental conditions [146].

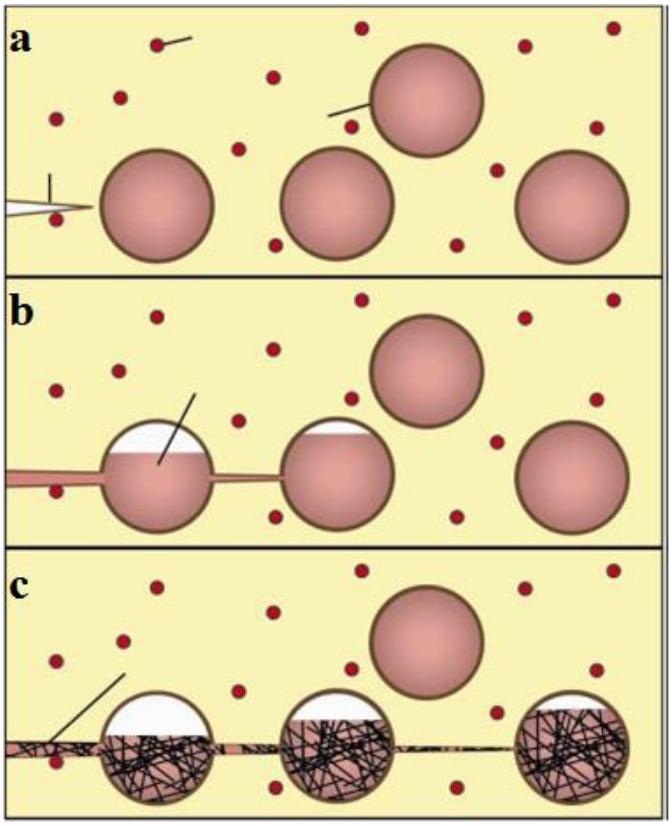


Figure 3- The autonomic healing concept. A microencapsulated healing agent is embedded in a structural composite matrix containing a catalyst capable of polymerizing the healing agent. a. Cracks form in the matrix wherever damage occurs; b. the crack ruptures the microcapsules, releasing the healing agent into the crack plane through capillary action; c. the healing agent contacts the catalyst, triggering the polymerization that bonds the crack faces closed. Reprinted by permission from Macmillan Publishers Ltd: [146].

3.4.6 Others

Microcapsules find broad application in the case of carbonless copy paper. Sheets of carbonless paper are coated with microencapsulated dye or ink and/or reactive substances.

Microcapsules, upon pressure-induced rupture, spill a dye (in colourless form in the capsules) and a colour developer that when mixed react to generate a colour. The rupture can occur under writing pressure or during printing using dot-matrix or impact printers. Many companies such as NCR Corporation, Appleton and Xerox supply this kind of paper. Numerous patents are available for carbonless copy paper, also known as pressure-sensitive recording papers [147-156].

Metallic particles have been widely studied as they present an interest both for fundamental physics and potential applications such as catalysis and magnetic recording [32], which can be prepared by using complexing microcapsules obtained by interfacial polycondensation. Laguecir et al. [26, 29, 157] studied the application in the extraction of metal ions. Given that polymeric microcapsules are porous, the wall is capable of physically absorbing some particles. On the other hand, the metal ions extraction is due to the complexion with polymers, such as PEI, both in the membrane and in the core [7]. As illustrated in Figure 3-1, this type of PEI-SC microcapsules contains non crosslinked PEI, being that the PEI molecules are too large to release from the shells. Therefore, PEI exists in both membranes and the core of microcapsules.

Many of interesting applications were disclosed by patents. US patent 20090029140 describes the process for treating substrate, preferably flexible substrate such as leather, leather imitations or polymeric films and textile substrate [158]. Cationic polymer and encapsulated materials may be used, for example, in a fabric softener composition [159]. Poly(vinyl alcohol) microcapsules and phenolic resin were used as wall materials to

encapsulate biocide comprising an isothiazolone biocide or antifouling agent, which is useful in marine antifouling coatings and paints [160].

## 3.5 **Microencapsulation for paper modification in our group**

Bioactive papers are obtained from the modification of the bulk or the surface of paper substrates with biomolecules with the goal of adding functionality to the paper. These functionalities may include detection, capture or inactivation of specific targets, such as harmful molecules or pathogens. These features of bioactive paper are designed to operate without using any sophisticated equipment or apparatus, making the device portable and adaptable to various uses and situations. The concept of bioactive paper thus far has been mainly oriented to fulfil specific needs in health, safety and food packaging applications. In our group, bioactive papers have been fabricated by the immobilization of different microencapsulated enzymes either through coating them onto the paper or by incorporation into the paper pulp. The physical processes of obtaining microencapsulation are the emulsion and encapsulator method for the same chemical process, namely as interfacial polycondensation. PEI microcapsules were prepared by interfacial polycondensation due to the benefits of this method, as mentioned in the introduction [17, 109, 113, 161-163].

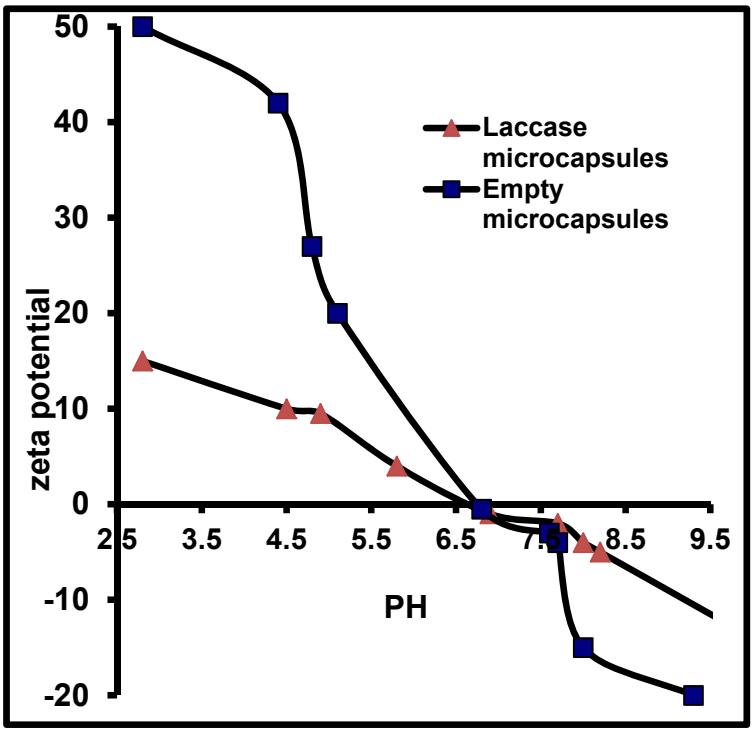


Figure 3- Zeta potential of empty (squares) and laccase (triangles) microcapsules at different pHs of the buffer solution.

Figure 3-6 illustrates the zeta potential of empty and laccase microcapsules prepared by the emulsion method at different pHs of the buffer solution. Our results showed that the absolute value of zeta potential decreased due to the encapsulation of enzymes, revealing that membranes of enzyme microcapsules have different properties with empty microcapsules. The dispersion of enzyme microcapsules is poorer than empty microcapsule due to the low zeta potential indicated in Figure 3-6, yet their isoelectric point is practically overlapping. Zeta potential is a function of the surface charge of the particle. If all the microcapsules in suspension have a large negative or positive zeta potential they tend to repel each other, hence there is no tendency to flocculate. However, if the microcapsules

have low zeta potential values, then there is no force to prevent the particles agglomerating and flocculating. As shown in Figure 3-6, the zeta potential can be improved by changing the pH of dispersant medium. Thereafter, the dispersion of microcapsules can be adjusted according to the preference of the core phase. For example, the optimal pH of microencapsulated laccase is 4.5, thus a suitable acidity solution, such as citric acid, can be used to wash and disperse produced microcapsules.

Bioactive papers prepared by the immobilisation of PEI microcapsules presented a higher retention of the enzyme on the paper sheets due to the size of the capsules and favourable electrostatic interactions between paper and microcapsules [113]. The enzymatic activity was also maintained for a longer period of time in the paper compared to free laccase, due to the protection from bacteria and proteases offered by the semi-permeable membrane [164]. The scalable synthesis of microcapsules offered by interfacial polycondensation is one of the great benefits of the technique which allows the modification of large areas of paper substrates which can be achieved either by coating or printing [162, 164].

## 3.6 **Conclusion**

Microencapsulation by interfacial polycondensation as a conventional method has been extensively studied, and the goal of this paper was to review some of the numerous applications of this technique. This approach offers a broad variety of opportunities such as protection and masking of fragile active ingredients, facilitation of handling and controlled release. The present article provided a literature review of different characterization

methods and potential application fields of microcapsules prepared by interfacial polycondensation method; it also provided core materials and physical properties of shells. Current research is focusing on the development of novel shell and core materials, and overcoming its obstacles, which may lead to many new applications. Microencapsulation of self-healing materials and PCMs are likewise receiving much attention.

**List of acronyms**

| Acronym | Full name | Acronym | Full name |
|---|---|---|---|
| ABTS | 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) | PAPI | Polyamine-polyisocyanates |
| AC | Acid chloride | PCM | Phase change material |
| AFM | Atomic force microscopy | PEI | Polyethyleneimine |
| CDEMA | 2-chloro-N-(2,6-diethylphenyl)-N-(methoxy methyl)acetamide | POPA | polyoxyalkylene polyamine |
| CLSM | Confocal laser scanning microscopy | PPD | p- phenylenediamine |
| DBDPO | Decabromodiphenyl ether | PTC | Phtaloylchloride |
| DBS | Dibutyl sebacate | PTFE | Polytetrafluoroethylene |
| DEG | Diethylene glycol | PU | Polyurethane |
| DETA | Diethylene triamine | SC | Sebacoyl chloride |
| DHEA | Dehydroepiandrosterone | SEM | Scanning electron microscopy |
| EDA | Ethylenediamine | SMA | Styrene–maleic anhydride |
| EGDE | Ethyleneglycol diglycidylether | TC | Trimesoyl chloride |
| FITC | Fluorescein isothiocyanate | TCMP | (Trichloromethyl)pyridine |
| HAD | 1,6-Hexamethylenediamine | TDC | Terephthaloyl dichloride |
| HMDA | 1,6-hexamethylenediamine | TDI | Toluene-2,4-diisocyanate |
| HMDI | Hexamethylene-1,6-Diisocyanate | TDITC | Terephthaloyl diisothiocyanate |
| HPVA | Hydrolyzed polyvinyl alcohol | TEM | Transmission electron microscopy |
| HSA | Human serum albumin | TEOHS | Tetradecaethoxyhexasiloxane |
| IPDI | Isophorone diisocyanate | TPC | Terephthaloyl chloride |
| MCR | Monocrotophos | TMP | Trimethylolpropane |
| MDI | Diphenylmethane-4,4'-diisocyanate | UF | Urea-formaldehyde |
| OES | Oligoethoxysiloxane | UG | Urea-gluteraldehyde |
| OPD | o-phenylenediamine | | |

**Acknowledgment**

The authors thank the financial support from the SENTINEL Research Network on Bioactive Papers and from the Natural Sciences and Engineering Research Council of Canada (NSERC).

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# Chapter 4 Comparison of emulsion and vibration nozzle methods for microencapsulation of laccase and glucose oxidase by interfacial reticulation of poly(ethyleneimine)[4]

## 4.1 Abstract

Microcapsules for enzyme immobilization were successfully fabricated via interfacial cross-linking of poly(ethyleneimine) (PEI). A method based on laminar jet break-up technique using a commercial instrument developed to produce alginate beads is reported for the first time for production of PEI microcapsules. The diameter, wall thickness and pore size of membranes were obtained from confocal laser scanning microscopy (CLSM) by labelling PEI and proteins. The composition of membranes was analyzed by elemental analysis. Larger microcapsules (ca. 200 μm diameter) were obtained with the encapsulation device. In comparison, the emulsion method produced smaller capsules (ca. 20 μm diameter) but with a wider size distribution. Encapsulation efficiency for both methods was analyzed by bincinchoninic acid (BCA™) and fluorescence assays, yielding efficiencies of 94±2% and 83±3% for the emulsion method and encapsulation device, respectively.

[4] This chapter was published as a paper in Journal of Microencapsulation.

Yufen Zhang, Dominic Rochefort, Comparison of emulsion and vibration nozzle methods for microencapsulation of laccase and glucose oxidase by interfacial reticulation of poly(ethyleneimine), Journal of Microencapsulation, 27(8), 2010, P703-713.

Glucose oxidase from *Aspergillus Niger* (GOx) and Laccase from *Trametes Versicolor* (TvL) were encapsulated by both microencapsulation methods and their activities were compared.

## 4.2 Introduction

Enzyme immobilization continues to hold significant promises for a broad range of applications. Biosensor is one of the most active research areas in technique development for enzyme immobilization since major improvements in the actual techniques on biosensors will lead to their greater application in the future.[1-3] Recent developments in biosensors also show the need in applying enzyme immobilization techniques on unconventional supports like plastics and papers. The impetus for such orientation is the application of biosensors to a larger public and to regions with difficult or inexistent access to advanced measurement technologies. As a result, we and others have carried out research on bioactive papers and paper-based biosensors.[4-8] Paper is already widely distributed and found uses in numerous hygiene, personal and packaging products, offering an unequalled potential to broaden the application of biosensors. Modifying papers with enzymes have applications in monitoring of glucose or protein levels in urine[6], assessing water quality and packaging to improve preservation[9, 10]. These appealing applications require novel enzyme immobilization techniques that will address the problem of enzyme stability while being compatible with current paper making processes for large-scale production.

We have oriented our effort towards developing an enzyme immobilization based on microencapsulation which consists of entrapment of biomolecules in the core of hollow spheres made by a semi-permeable membrane, thus providing a chemically and mechanically robust system. Once prepared, the capsules can be easily incorporated into or onto paper sheets by simple mixing with pulp or by deposition on the surface of the sheet.[4] Microcapsules can also be coated or printed on paper to make it compatible with current high-end papermaking technologies. In this paper PEI is studied as a wall material to produce microcapsules containing hydrophilic cores. PEI is a cationic polymer commercially available in a broad range of molecular weights (200–800,000 Da) with different degrees of branching.[11, 12] The good biocompatibility of PEI already makes it appealing for many applications in biotechnology. It is one of the most frequently used polycations for the condensation of DNA[11-13] and for entrapment of biocatalysts and drugs due to its compatibility with most enzymes, cells and natural compounds.[14] PEI microcapsules can be prepared efficiently from the fast interfacial condensation of the polymer with a diacid chloride. During the cross-linking reaction, the primary and secondary amines on the highly-branched PEI react with the acid group at each end of the sebacoyl (decanedioyl) chloride through nucleophilic substitution reaction to covalently linking the chains together. The first and the crucial step in PEI microcapsules preparation is the formation of stable microdroplets of the aqueous phase containing the polymer. This is normally achieved by emulsifying a PEI solution (aqueous phase) in an organic solvent prior to the addition of the cross-linking agent. The broad range of microdroplet diameters obtained from such emulsion methods results in microcapsules with a large size

distribution. In order to achieve a better control on the microcapsule formation and to reduce their size distribution, we have adapted a commercial encapsulation device used for cell encapsulation in alginate beads for the formation of PEI microcapsules by interfacial reticulation.[15, 16]

The present article will focus on the comparison of both microencapsulation methods and the characterization of microcapsules. The factors that can impact size, membrane thickness and composition of microcapsules will be described. PEIs of three different molecular weights were used to prepare microcapsules by these two approaches to study the differences in properties of the capsules with respect to permeation across the membrane.

## 4.3 Materials and methods

### 4.3.1. Materials

Sebacoyl chloride (SC), cyclohexane, Span 85, Sulforhodamine 101 acid chloride (Texas red) and fluorescein isothiocyanate (FITC), bovine serum albumin (BSA), TvL (EC 1.10.3.2), GOx (E.C. 1.1.3.4) and QuantiPro BCA (bicinchoninic acid) assay kit were purchased from Sigma-Aldrich. Salts used for buffer preparation were purchased from A&C American Chemicals Ltd and MilliQ water (18 MΩ·cm) was used in the preparation of all solutions. All three PEI aqueous solutions (50 wt. %) with the average molecular weights (MW) of 1 300, 2 000 and 750 000 Da were purchased from Sigma Aldrich and

used as received. For convenience, these different PEI will be referred to as PEI1300, PEI2000 and PEI750k, respectively.

4.3.2. Preparation methods of PEI microcapsules

Two methods were used to prepare microcapsules via interfacial polycondensation of PEI. In the mechanical emulsion method, a microemulsion of 10ml McIlvaine buffer (aqueous phase, pH 8.0) containing PEI (4.8 % v/v) and 1% v/v of Span 85 was achieved in 50ml cyclohexane (continuous phase) for 15min with a 4cm of 4 blade axial impeller stirrer. SC was then added to the emulsion to crosslink PEI at the interface of hydrophilic microdroplets, which was conducted in a 250ml beaker. This is a updated method based on the first description of PEI-SC microcapsules reported by Poncelet et al.[17] Enzyme and BSA encapsulation was obtained by dissolving the proteins in the aqueous phase prior to its emulsification in the organic phase.

The second technique (which will be herein referred to by the name encapsulator) is based on the laminar jet break-up technique and is performed with a commercial encapsulation device (Inotech IE-50R Encapsulator). A detailed description of the instrument and its use for the production of alginate beads can be found in references [15, 16]. A typical batch of capsules was prepared with this technique by pumping a solution (10 ml) of McIlvaine buffer (pH 8.0) with 5% v/v of PEI through the nozzle while applying a vibration at high frequency. The aqueous microdroplets formed at the nozzle were then collected in a stirred reaction vessel containing 50 ml of cyclohexane with 1% v/v Span85

as an emulsifier and 0.8% SC as the cross-linker. The capsules obtained were incubated for 5 minutes in the organic phase to mature the membrane before stopping the reaction by dilution with 50 ml of cyclohexane. The suspension was allowed to settle for 1 minute and the supernatant was discarded. Microcapsules were rinsed twice with 100 ml cyclohexane to remove unreacted organics and surfactants, transferred in filter funnel and thoroughly rinsed again with MilliQ water. Figure 4-1 presents a scheme describing microcapsule formation by both methods.

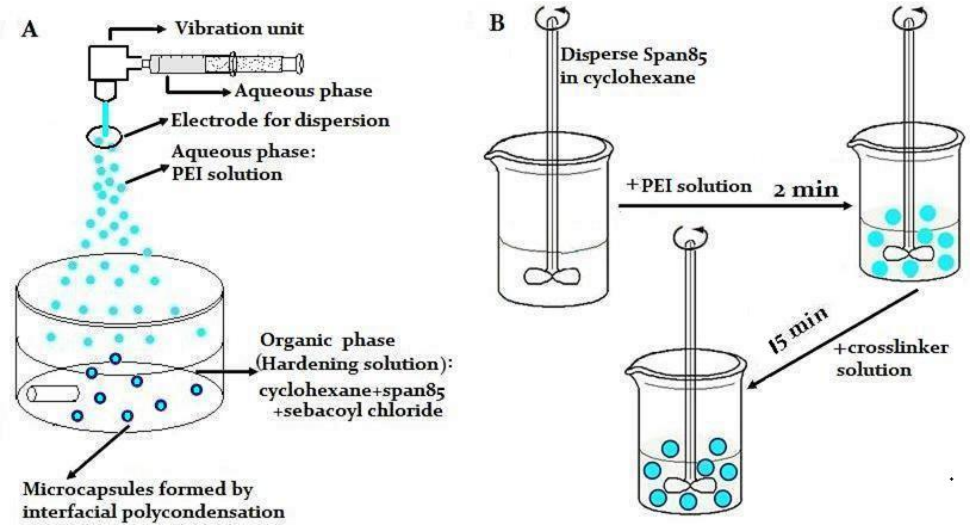


Figure 4- Schematic diagrams of microcapsule formation with the encapsulation device (A) and by the emulsion method (B).

### 4.3.3. Characterization of microcapsules

A direct, microscopic approach was used to determine the wall thickness of microcapsules. PEI samples with different MW were first labelled with the fluorescent tag

FITC and then dialysed using a filtration membrane (2 kDa MW cut-off) to remove any unreacted fluorophores. Labelled PEI samples were then used to prepare microcapsules as detailed above and the resulting fluorescent microcapsules were studied by Confocal Laser Scanning Microscopy using a Leica TCP SP5. The diameter of isolated microcapsules and their wall thickness were measured from the micrographs and histograms recorded at the centre of the capsules. As this technique only provides diameter for isolated microcapsules, a particle size analyzer (Horiba Partica LA-950) based on laser diffraction is also used to evaluate the average particle size and size distribution of thousands of microcapsules.

The determination of the ratio between PEI and crosslinker SC in the microcapsules membranes was achieved by elemental analysis [18] and thermogravimetric analysis (TGA). EA was carried out in an elemental analysis instrument from Fisons Instruments SPA, Model EA1108. TGA measurements were made on a thermogravimetric analyzer, Universal V3.5B from TA Instruments, using a scanning rate of 10°C/min and curves were recorded within a temperature range of 25-600°C. All analyses were performed under a nitrogen-gas atmosphere in platinum crucibles. The freshly-prepared microcapsule samples were first dried at room temperature and then left at 60°C for 8 h in a vacuum oven. Alternatively, for the samples made from PEI1300 and PEI2000, water was removed by lyophilisation. The samples were fast frozen in acetone-dry ice bath prior to be dried with VirTis frezemobile 25EL sentry 2.0 lyophilizer, and the condenser was set to -86°C. In elemental analysis, carbon and nitrogen contents in the reagents and the membranes were obtained, and then the ratios between nitrogen and carbon elements in the reagents and the

membranes were used to calculate the final results which were further confirmed by TGA analysis.

The porosity of PEI microcapsules was determined under wet conditions with the capsules suspended in a buffer solution. The molecular weight cut-offs of PEI membranes were estimated by their ability to diffuse across the capsule wall, which was determined by confocal microscopy. To do so, PEI (1 300 or 2 000 Da) and Laccase (66 000 Da) were used as MW beacons and labelled with FITC in 0.1 M carbonate buffer (pH 9) at 4°C in the dark for 6 h. The FITC-PEI solutions were then dialysed with regenerated cellulose membranes (Spectrum Laboratories, cut-off 1kDa) against 250 ml of MilliQ water for 7 days (water was changed twice a day) in the dark at 4°C to remove any uncoupled FITC. Laccase was also tagged with FITC in water at 4°C in the dark for 4h, but in this case, unbound FITC was removed by gel chromatography.[19] In order to distinguish the above MW beacons from the microcapsules, their membranes were labelled with a different fluorescent tag. The microcapsules were reacted with Texas-red (sulforhodamine 101 acid chloride) in pH 9 Tris-Cl buffer at 2°C in the dark for 4 h and with a labelling ratio of 2% $w_{dye}/w_{capsules}$.[19] Unbound or hydrolyzed Texas-red was removed by plenty of rinsing water, until the dye became undetectable in the water. The Texas-red tagged microcapsules were then suspended in a solution of each MW beacons and left at 4°C for 16 h to allow equilibration between the inner (core) aqueous phase of the microcapsules and the surrounding solution. Samples were then removed from the MW beacon solutions and confocal microscopy was used to examine the capsules.

Entrapment efficiency of both encapsulation techniques was measured with the BSA protein since it has a MW similar to that of TvL. During BSA encapsulation, solution from both the reaction vessel and rinsing solutions were collected for protein content assay using QuantiPro BCA assay. Prior to this, free PEI in the core which interferes with the measurement was removed by a 30 kDa MW cut-off membrane. The use of high molecular weight (750 kDa) PEI made it difficult to separate the unencapsulated proteins from a solution containing the large polymer. Therefore, a different assay was used for the efficiency of the encapsulator method, in which BSA was conjugated with FITC prior to its encapsulation and the solutions were measured by fluorescent signal using a spectrofluorometer (Infinite 200).

## 4.4 Results and Discussion

### 4.4.1 Microencapsulation with an encapsulation device

Microcapsules of high quality can be produced by interfacial condensation of HMW PEI using the encapsulation instrument described in section 4.2.2. This encapsulator is commercialized for the scalable production of alginate beads for living cells immobilization. [15] Here we applied this instrument to generate microdroplets from an aqueous solution of PEI instead of alginate to prepare hollow microcapsules. To do so, the calcium hardening solution was replaced by a cyclohexane phase containing a cross-linking agent to achieve interfacial polycondensation with PEI. In comparison with the emulsion method (Section 4.2.2.), the encapsulation device offers more flexibility on the size of the

microdroplets by adjusting the nozzle and its parameters. Since these instrumental parameters are dependent on the viscosity and surface tension of the shell forming phase (here aqueous PEI solution), the same polymer and concentration are used in a sequence of experiments to determine the effect of these instrumental parameters. Bead formation is achieved by breaking up the laminar jet created at the nozzle of the instrument with a mechanical vibration applied to the fluid.[16] In general, the nozzle diameter has the strongest influence on the microcapsule size given that the beads formed with this technique have a diameter approximately twice of the nozzle diameter. When the instrument was equipped with a 100 μm nozzle, flow rate of aqueous phase was found to be the most important parameter affecting the size of microcapsules. The diameter of microdroplets is affected to a negligible extent by the frequency of the vibration. Amplitude of the vibration waveform and magnitude of the electrostatic charge did not affect the mean diameter of produced microcapsules. Only a narrow range of parameters allow formation of spherical and stable microcapsules but, generally, mean diameter of microcapsules will be affected by about ±20% by varying pump speed. Figure 4-2 A for instance presents the impact of flow rate on the mean diameter of capsules. The size of the microcapsules decreased as the flow speed increased. Flow rates above 4.4 $ml.min^{-1}$ produced many particles in addition to the microcapsules and those were mostly of irregular, non-spherical shape. This can be explained by the pressure that builds up in the nozzle and disrupts the continuous flow of PEI solution. Low MW PEI (1 300 and 2 000 Da) solutions present a lower viscosity, therefore allowing higher flow rates (up to 5.6 $ml.min^{-1}$) to pass through, resulted in microcapsules with smaller mean diameters (108 μm as seen in Figure 4-2 B). A

smaller viscosity increases the optimal frequency of the vibration waveform required to break the laminar flow in monodispersed microdroplets, which in turn reduces the diameter of the droplets. However, microcapsules made from LMW PEI had spherical shapes before washing, but were destroyed partially after washing.The concentration of cross-linker SC in the cyclohexane phase affects the microcapsule formation by achieving a higher degree of reticulation which results in thicker membranes. This effect of cross-linker concentration on the capsule formation by the encapsulation device is similar to what have already been observed with an emulsion-based method.[17] To evaluate this effect in our system, we varied the cross-linker concentration from 0.2 to 1.6 v/v %. While a low concentration yielded fragile capsules due to their thinner membrane, a high concentration increased the agglomeration of the microcapsules. The surfactant stabilizes the aqueous microdroplets in the cyclohexane, therefore preventing their agglomeration during the cross-linking reaction and membrane formation. We have determined that the concentration range of 1 to 2 v/v % is the most suitable for microcapsule formation with the encapsulation device.

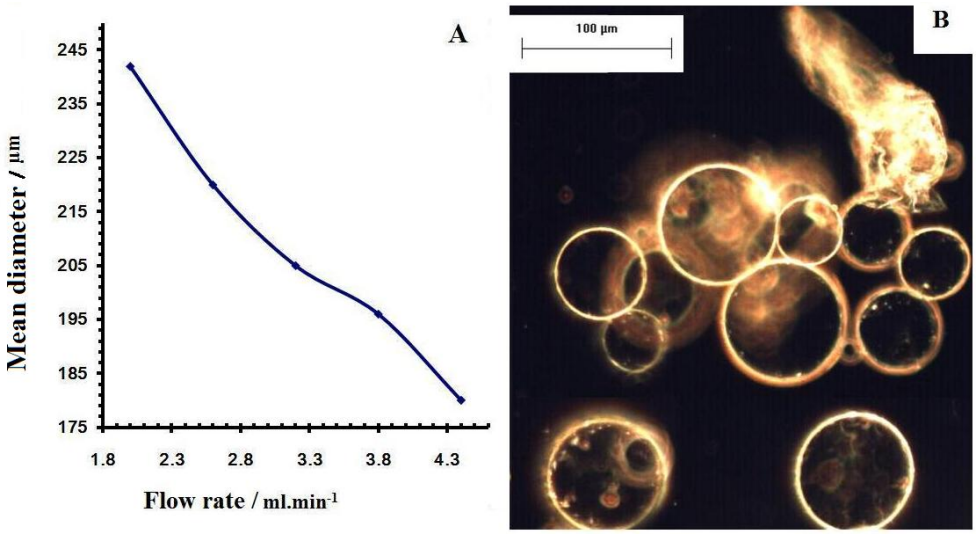


Figure 4- Effect of the flow rate at the nozzle of the encapsulation device on the microcapsules mean diameter (A) and optical micrograph of irregular microcapsules obtained at high flow rates (B).

Other parameters can be adjusted on the encapsulation device to improve microdroplet formation and dispersion but were found to have only very limited effect on the size of the microcapsules. The frequency and amplitude of the vibration waveform must be adjusted to provide uniform chains of well-dispersed microdroplets. In addition, a ring-shaped electrode (see Figure 4-1) allows electrostatic charging the microdroplets and the voltage applied on this electrode can be adjusted to improve dispersion. All these parameters were varied independently and the values selected to produce microcapsules with the lowest size distribution, less agglomeration and highest stability are listed in Table 4-1. The coefficient variation (CV) of produced microcapsules can go down to 18% under optimal parameters.

Table 4- Optimal reagent ratios and instrumental parameters for preparation of PEI microcapsules with the encapsulation device.

| PEI concentration | S.C. concentration | Emulsifier concentration | Voltage /kv | Frequency /Hz | Flow rate/ $ml.min^{-1}$ |
|---|---|---|---|---|---|
| 5% (v/v) | 0.4%-1.0% (v/v) | 1% (v/v) | 0.9-1.8 | 800-3000 | 2.6-3.4 |

### 4.4.2 Characterisation of microcapsules

#### *4.4.2.1. Size and size distribution*

Microscopy was used to compare the size, size distribution and aspect of microcapsules prepared by the two methods. Figure 4-3 shows microcapsules made by the emulsion method (A and B) and by the encapsulator (C and D). As optical microscopy provided only limited information on the small capsules obtained by emulsion due to their size, images A and B were obtained from fluorescence micrographs (CLSM). As expected, the emulsion method yielded small capsules (5 to 50 μm diameter) with a very broad size distribution. The mean diameter of microcapsules could be varied from 15 to 40μm by adjusting the impeller rotation speed from 775 to 2130 rpm. Figure 4-3A shows most of the smaller microcapsules tend to aggregate more than the larger ones due to surface tension effects. PEI with a low molecular weight (1 300 Da) is preferably used to produce microcapsules with the emulsion method (Figure 4-3A and B) since those obtained with high molecular weight PEI (750 kDa) using the same method are strongly aggregated and showed an irregular shape. Low molecular weight PEI is insoluble in cyclohexane [20] but the longer

chains of high molecular weight PEI, on the other hand, might protrude from the interface to some extent or reach a neighbouring microdroplet. Using HMW PEI can however be advantageous for the preparation of larger capsules by allowing the formation of thicker and stronger membrane walls. Figure 4-3C and D present images obtained by optical microscopy that are representative of the microcapsules prepared by the encapsulator method described in the experimental section. The microcapsules seen in Figure 4-3 were prepared from high (C) and low (D) MW PEI. One can first notice that the capsules are much larger than those presented in A and B, with an average diameter of 210 μm and with a smaller size distribution. Adjusting the parameters described in Section 4.3.1 provided capsules with mean diameters ranging from 180 to 260 μm. Using this method, high MW PEI produced well-dispersed capsules that maintained a spherical shape. On the contrary, when LMW PEI is used, the capsules appeared fragile and collapsed after a short period of time (Figure 4-3D). HMW PEI capsules present stronger walls than their LMW counterparts due to the denser network of reticulated PEI achieved by much longer polymer chains (2 vs. 750 kDa).

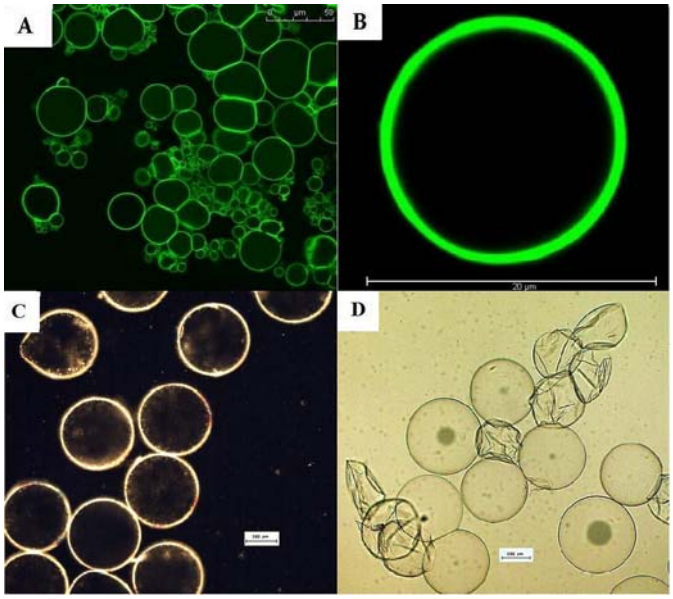


Figure 4- Micrographs of typical microcapsules prepared by the emulsion method (A and B) and by the encapsulation device (C (PEI750k) and D (PEI1300)). The micrographs A and B were obtained by CLSM using FITC-modified PEI.

Vibration nozzle method can produce larger microcapsules than emulsion method (Figure 4-4) but with a narrower size distribution. As shown in Figure 4-4, the mean diameter of microcapsules prepared by emulsion method at rotation speed of 1140 rpm is about 25 µm while the mean diameter of microcapsules prepared by vibration nozzle method are much larger: about 202 µm at pump speed of 3.8 $ml.min^{-1}$.

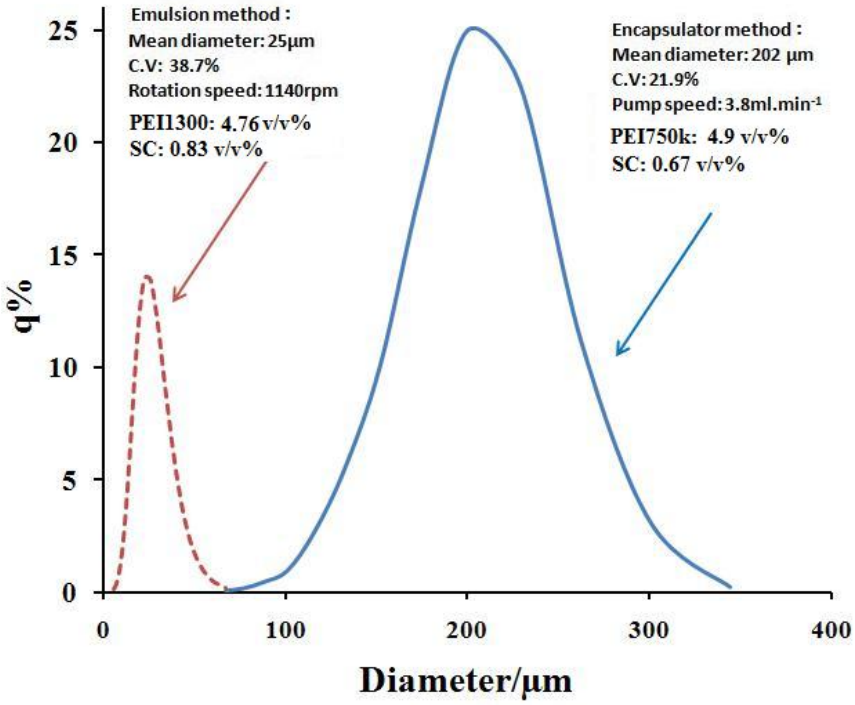


Figure 4- Size distribution of microcapsule prepared by emulsion method (dashed line) and vibration nozzle method (solid line). The data was obtained from particle size analyzer based on laser diffraction (Horiba).

*4.4.2.2. Wall thickness*

Several techniques, including surface plasmon resonance spectroscopy [21, 22], ellipsometry [23], differential scanning calorimetry [24], atomic force microscopy [25, 26], transmission electron microscopy [25] and CLSM [26], have been used to determine the wall thickness of several types of microcapsules. Here, the process of determining microcapsules membrane thickness was carried out by CLSM, using FITC-modified PEI in the preparation of the capsules. The wall thickness is easily measured from the histograms

of the cross-section of the microcapsules (Figure 4-5 A and B). This technique is however only relevant to the microcapsules prepared from low MW PEI (1 300 and 2 000 Da) as the labelled PEI which did not participate in the membrane formation was removed from the inner core of the capsules during the washing step of the preparation procedure. The microcapsules prepared with the encapsulation device from high MW PEI presented a uniform fluorescent signal throughout all of their cross-section (Figure 4-5 C), making the membrane thickness difficult to evaluate. The uniform fluorescent signal was due to the remaining labelled PEI which could not be removed from the core of the capsules during the washing step because of its large size (molecular weight of 750 kDa). Breaking the microcapsules allowed high MW PEI to be released from the capsules and the remaining signal was solely attributed to the membrane (Figure 4-5 D). The wall thickness of capsules made from high MW PEI by the encapsulator method at a flow rate of 3.2 $ml.min^{-1}$ was about 4±2 μm. In comparison, the wall thickness of capsules made from PEI1300 and PEI2000, using the mechanical method at 1640 rpm, were only about 1.5±0.8 and 2±0.9 μm, respectively. As expected, increasing the PEI MW resulted in microcapsules with thicker membranes for a given amount of cross-linker because of the polycondensation of longer polymer chains. The mean thickness of the membranes increased with the concentration of cross-linker by allowing more PEI chains to participate in membrane wall formation. Increasing the concentration of crosslinker from 0.4% to 1.1% resulted in an increase of membrane thickness from 1.0 to 2.0μm. However, at cross-linker concentrations above 1.2 v/v%, the microcapsules presented irregular surfaces and most of them agglomerated.

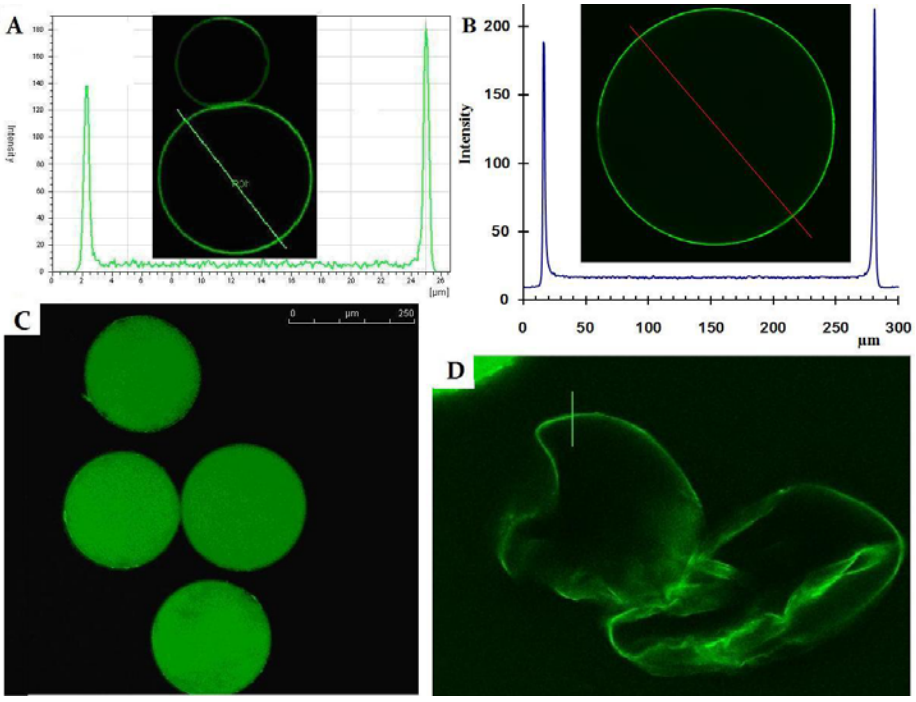


Figure 4- (A and B) Fluorescence intensity histograms calculated from the corresponding confocal micrographs of microcapsules (insets) prepared with FITC-tagged PEI (2 000 Da) using the emulsion method (A) and the encapsulation device (B). Images in C and D are micrographs obtained for microcapsules prepared by the encapsulation device with high MW PEI (750 kDa). Image in D shows how broken microcapsules are emptied of the fluorescent polymer.

### *4.4.2.3. Membrane composition*

Wet microcapsules contained approximately 96.8% water, which was analyzed by lyophilisation of wet microcapsules. The moisture environment in the microcapsules could keep the native structure of microencapsulated enzymes.

The results of the elemental analysis presented in Table 4-2 were obtained from microcapsules prepared by both methods using various concentrations of the SC cross-linker with a fixed amount of PEI. The ratios between PEI and SC were then calculated from the relative proportions of carbon and nitrogen which changes upon reticulation because SC does not contain any N atoms. The carbon and nitrogen content of PEI measured by EA are given in the bottom lines of Table 4-2. One can readily notice from Table 4-2 that increasing the concentration of SC from 0.33 to 0.83% resulted in insignificant changes in the membrane composition for both methods and with both molecular weights of PEI. This is in agreement with our observation that membrane wall thickness slightly increases with the cross-linker concentration. Table 4-2 also shows that microcapsules formed by PEI with a MW of 2 000 Da present a slightly higher proportion of the polymer in their membranes which can be explained by the reduced pore size obtained with longer polymer chains. Smaller pore size resulted in a lower diffusion rate of unreacted polymer which was trapped in the membranes and therefore increased the ratio of PEI found in the capsules. This is further exemplified by the higher amount of PEI found in the membranes of microcapsules formed with the encapsulator with 2 kDa PEI which present thicker membranes than those obtained from the emulsion method. As a result of this PEI entrapment effect, the membranes of microcapsules made with 750 kDa PEI could not be analysed by EA because most of the polymer was retained inside of their core (Figure 4-5 C), resulting in an overestimation of the membrane content in polymer.

Table 4- Results from the elemental analysis of the microcapsule membranes composition prepared by the emulsion method and the encapsulation device.

| Method | PEI MW (Da) | Cross-linker (v/v %) | % N found | %C found | Calculated PEI:SC ratio |
|---|---|---|---|---|---|
| Emulsion method | 1300 | 0.33 | 18.1 | 53.1 | 56.2 : 43.8 |
| | | 0.50 | 17.1 | 50.7 | 55.7 : 44.3 |
| | | 0.83 | 17.2 | 51.6 | 55.3 : 44.7 |
| | 2000 | 0.33 | 18.2 | 50.7 | 59.9 : 40.1 |
| | | 0.50 | 18.2 | 51.0 | 59.9 : 40.1 |
| | | 0.83 | 17.8 | 51.4 | 58.1 : 41.9 |
| Encapsulation device | 1300 | 0.33 | 17.9 | 55.4 | 56.7 : 43.3 |
| | | 0.50 | 18.7 | 54.5 | 56.7 : 43.3 |
| | | 0.83 | 18.7 | 54.5 | 53.5 : 46.5 |
| | 2000 | 0.33 | 19.9 | 52.9 | 66.6 : 33.4 |
| | | 0.50 | 20.6 | 52.4 | 65.9 : 34.1 |
| | | 0.83 | 20.8 | 52.4 | 63.1 : 36.9 |
| | 1300 | - | 30.2 | 49.8 | - |
| | 2000 | - | 29.6 | 49.6 | - |

Figure 4-6 represents the TGA curves obtained for microcapsules prepared from 2 kDa PEI with three different concentrations of SC. The solid line represents PEI alone to verify the incidence of the cross-linking reaction on the TGA curves. One can notice a 50°C shift toward higher temperature for the first weight loss around 100°C which corresponded to

water evaporation. PEI and microcapsules are highly hydrophilic and can take up moisture from air. This moisture requires more time and energy to leave the microcapsules where it is incorporated within the membrane wall. The effect of cross-linking was also evidenced by the apparition of a slope between 400°C and 475°C that was not present on the curve for PEI and which represented the decomposition of functional groups created during the reaction between the diacid chloride and the polymer. Similarly to Table 4-2, higher amounts of cross-linker did not affect significantly the chemical composition of the microcapsule membranes.

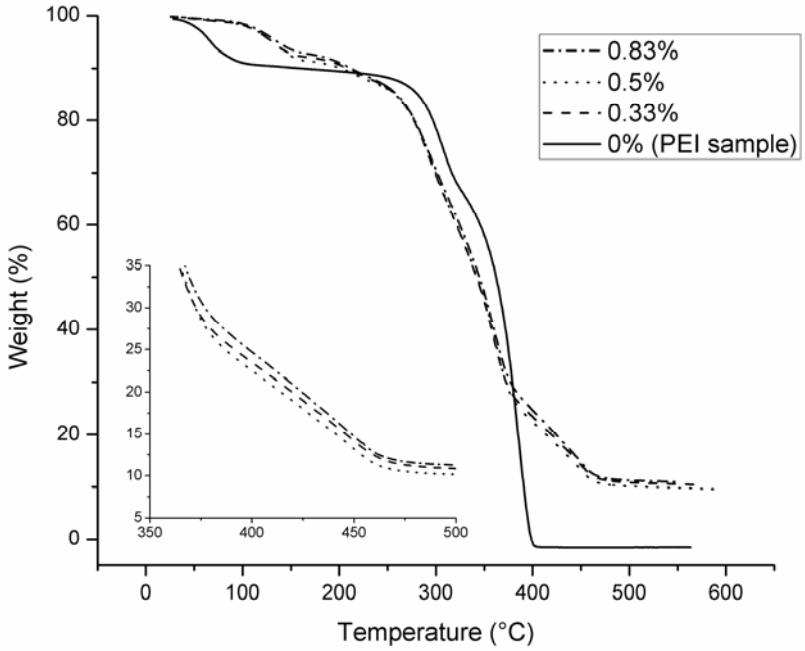


Figure 4- TGA curves of microcapsules prepared by the mechanical method with 2000 Da PEI and various concentration of cross-linking agent (v/v % values are listed in the legend). The solid line was obtained for the PEI polymer alone. Inset is the amplification of the apparition from membrane decomposition slopes between 375°C and 500°C. All experiments were conducted at a heating rate of 10 °C/min in a $N_2$ environment.

#### *4.4.2.4. Membrane cut-off*

Porosity is a factor of great importance in microencapsulation. Since our goal is to develop an efficient enzyme immobilization platform for biosensors, the microcapsules must have the right porosity to avoid enzyme leaching while allowing a quick diffusion of the substrate and reaction products. Thermoporometry, a technique based on DSC measurements, has been successfully used to evaluate the porosity of alginate hydrogel beads.[27, 28] Its use to determine the porosity of the thin membrane of hollow-core microcapsules filled with water however remains to be demonstrated. We therefore relied on a simple method based on confocal fluorescent microscopy to evaluate the diffusion of molecules of various molecular weights into the microcapsules and measured the MW cut-off. In this method, the membrane of the microcapsules was modified with a red-emitting fluorophore (Texas red) while the molecular weight beacons were tagged with a green-emitting label (FITC). Solutions of three different MW beacons (PEI1300, PEI2000 and laccase from *T. versicolor* 66 kDa) were incubated with the tagged microcapsules for 16 h. The appearance of a green signal either in the core or in the membrane of the capsules was then used to establish the membrane MW cut-off (see scheme in Figure 4-7 D).

The confocal micrographs shown in Figure 4-7 are for microcapsules prepared with PEI of 2 kDa (red signal) either by the emulsion method (images A and C) or the encapsulation device (image B). The green signal of the MW PEI1300 and PEI2000 presented in the core of the capsules (Figure 4-7 A and B) showed that PEI could diffuse through the membranes of capsules prepared by both methods. Figure 4-7 C shows however that laccase enzyme

was too large to permeate through the membrane and its signal was found only on their membrane wall. Consequently, microcapsules prepared by either of the two methods provided an efficient retention of the enzyme while allowing the diffusion of substrates with molecular weights below 2 kDa. Most substrates of interest for our biosensors fall in this category with MWs of a few hundreds of Daltons. The porosity analysis can be refined by calculating the radius of hydration of the molecules used to carry out the microscopy experiment. PEI is a globular molecule for which the $r_H$ values can be calculated from the molecular weight. The radii of the PEI molecules used in this study were calculated from the equation $r_H = 0.068\ MW^{0.39}$ [29] and the hydrodynamic radius of PEI (2 kDa) was 1.3 nm. In addition, the dimensions of laccase were determined to be 65×55×45 $Å^3$, for an approximate diameter of 5 nm.[30] The MW cut-off of capsules membranes in their fully hydrated form was therefore found between 2.6 and 5 nm.

PEI-SC microcapsules are quite strong, they can be rehydrated for many times, keeping the same morphology. Although small changes in porosity after they were dried and rehydrated are possible, the end application requires only for small molecules to diffuse (substrate) and their access to the core should not be modified after drying and rewetting.

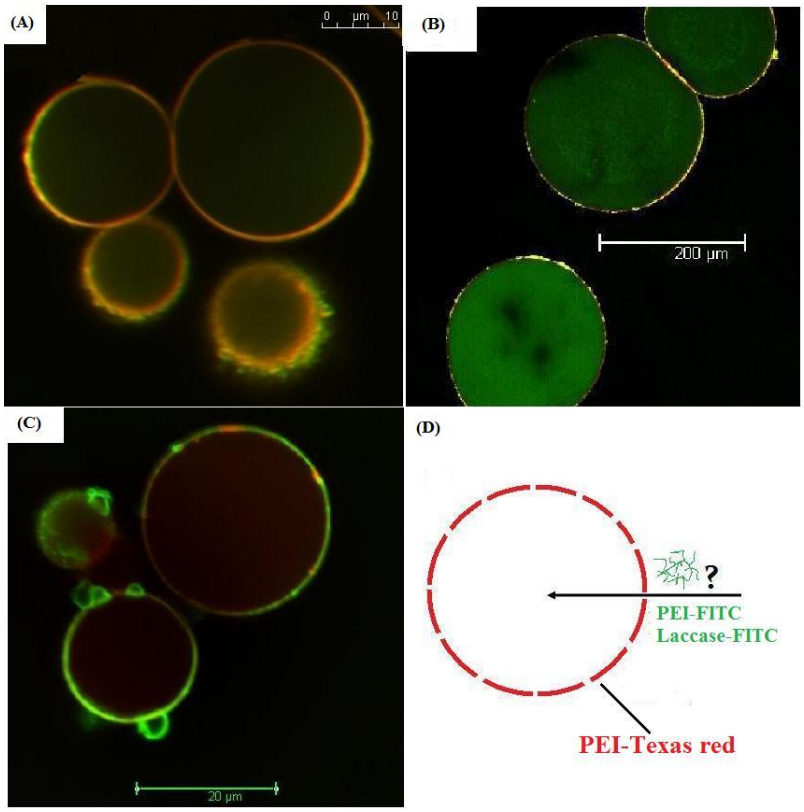


Figure 4- Confocal micrographs showing the combined signals of microcapsules membranes (red) and of different molecular weight markers (green). Microcapsules were prepared either with the emulsion method (A and C) or with the encapsulation device (B). The molecular weight markers labelled with FITC were PEI1300 (A), PEI2000 (B) and laccase from *T.versicolor* (C). The scheme in D shows a diagram explaining the experiment.

### *4.4.2.5. Encapsulation efficiency*

The encapsulation efficiency is herein defined as the percentage of proteins actually contained in the microcapsules relative to the amount initially used in the aqueous phase.

To evaluate the efficiency, a solution of the BSA protein was used in the preparation of microcapsules and the free BSA concentrations (i.e. that of the proteins that were not entrapped in the capsules) of all liquid phases, including those used to wash the microcapsules, were measured. This method therefore provided the amount of proteins that were tightly entrapped in the capsules as some located on the outer surface of the capsule membrane could be leached easily.[31] The emulsion method presented a higher efficiency (94±2%) compared to the method based on the encapsulation device (83±3%). For the same amount of reagents, emulsion method can produce more than double mass of microcapsules than encapsulator method, since microdroplets produced by emulsion method are much smaller. On the other hand, LMW PEI has low toxicity. Then emulsion method has better encapsulation efficiency than encapsulator method.

### 4.4.3 Enzyme microcapsules

Figure 4-8 represents the pH profile for the activity of free and encapsulated glucose oxidase obtained for the oxidation of glucose. The optimal pH of microencapsulated GOx showed a shift of 0.5 pH unit towards lower value comparing with free GOx. The proton exchange properties of PEI resulted in a membrane pH slightly different to the solution pH, affecting the enzyme activity in the microcapsules. The profile shows a broadening of the curve near maximal activity that was to be expected for an enzyme immobilized in a polymeric matrix.

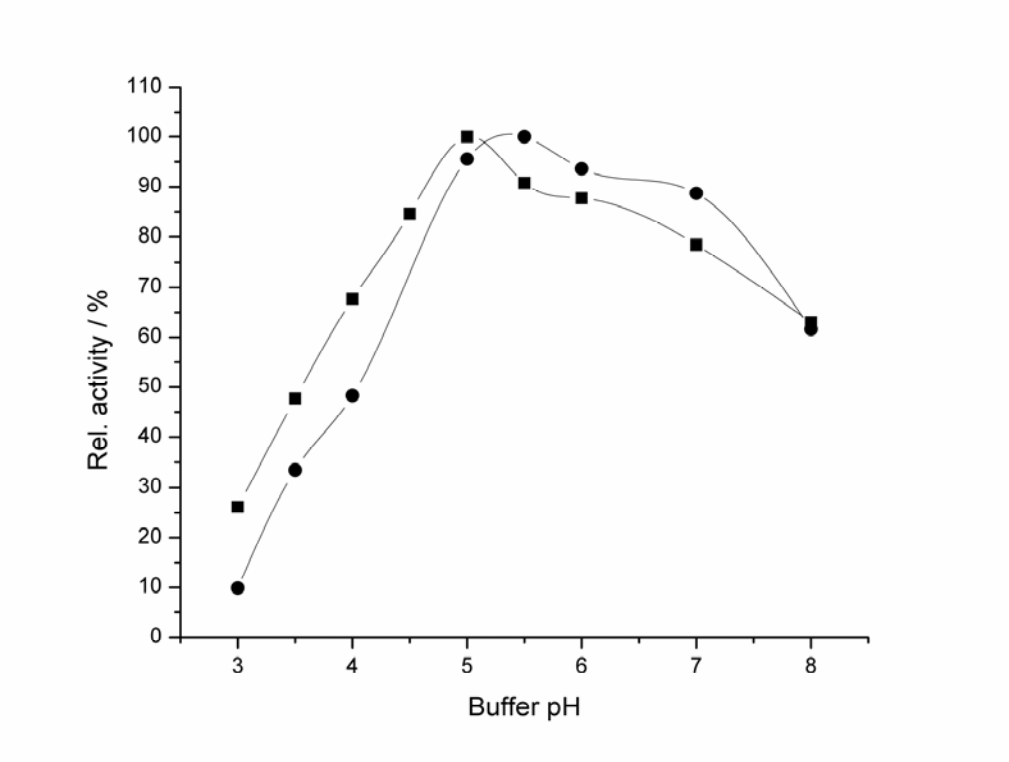


Figure 4- pH dependency of glucose oxidation by GOx either free in solution (squares) or in PEI microcapsules (circles). Glucose oxidation was monitored by oxygen consumption in McIlvaine buffers at 25°C.

The enzymatic activities of GOx and TvL microcapsules were found to be strongly dependent on the microencapsulation procedure as well as on the nature of the enzyme. The activity of TvL microcapsules prepared by emulsion method was 6.2 times higher than those prepared by encapsulator method, whereas the activity of GOx microcapsules prepared by emulsion method was only 2.4 times higher compared to the encapsulator method. Regarding the effect of the encapsulation method, the encapsulation device had lower encapsulation efficiency but the enzymatic activity losses were mainly due to the

higher toxicity of HMW PEI towards the enzymes compared to lower MW polymer. In addition, the thicker membranes produced with HMW PEI limited the substrate diffusion, resulting in lower enzymatic activity. HMW PEI also tended to be trapped inside the cores and could not be washed off easily due to its large size. Enzyme microcapsules prepared by encapsulator method therefore had a lower activity than those obtained with the emulsion method which yields better microcapsules with LMW PEI. The differences observed between the activities of microencapsulated laccase and glucose oxidase should be explained by a different enzyme-polymer interaction. Laccase appeared to be more sensitive to the presence of PEI than glucose oxidase. A detailed study of the PEI-enzyme interactions will be the topic of a forthcoming publication.

## 4.5 Conclusions

A commercial encapsulation device for the production of beads was used for the first time to prepare microcapsules from the interfacial reticulation of PEI. We have provided a methodological description of the formation and a characterization of PEI microcapsules prepared by an emulsion method and with the encapsulation device. Microcapsules can be prepared in mild conditions with high entrapment efficiencies under optimal instrumental parameters and optimal reagents ratios. The vibration nozzle technique produced microcapsules with a narrow size distribution with diameters of about 200µm. The characterization of microcapsules shows that the chemical reactions involved in the reticulation by the two methods are similar. PEI with large molecular weight must however be used to obtain stable microcapsules using the encapsulation device. In terms of the

characteristics of microcapsules, such as diameter, wall thickness, pore size, biocompatibility and entrapment efficiency, microcapsules prepared by emulsion method have better properties to entrap enzymes. Moreover, the smaller size of the microcapsules prepared by the emulsion method makes them more appropriate for an immobilization on paper using a conventional thin layer coating. Mechanical strength is an important factor for coating application, and is currently being investigated. The activity analysis of microencapsulated enzymes and the effect of the microencapsulation procedure on their structure will be discussed elsewhere.

## Acknowledgment

The authors thank the financial support from the SENTINEL Research Network on Bioactive Papers and from the Natural Sciences and Engineering Research Council of Canada (NSERC).

## Declaration of Interest statement

The authors report no declarations of interest.

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# Chapter 5 Activity, conformation and thermal stability of laccase and glucose oxidase in poly(ethyleneimine) microcapsules for immobilization in paper [5]

## 5.1 Abstract

In this paper we report on the microencapsulation of glucose oxidase from *Aspergillus niger* (GOx) and laccase from *Trametes versicolor* (TvL) with the goal of immobilizing these enzymes in paper substrates to develop biosensors and bioreactors. Despite having high encapsulation efficiency, the technique caused a severe decrease (up to 65%) in the specific activities of both enzymes once microencapsulated. Encapsulated TvL presented lower $K_M$ and $V_{max}$ values compared to free laccase in solution, but the $K_M$ for GOx did not change after microencapsulation. TvL was found to interact strongly with poly(ethyleneimine) (PEI) in a manner similar to that expected from an uncompetitive inhibition mechanism, and fluorescence and circular dichroism spectroscopy demonstrated

[5] This chapter was published as an article in Process Biochemistry.

Yufen Zhang, Dominic Rochefort, Activity, conformation and thermal stability of laccase and glucose oxidase in poly(ethyleneimine) microcapsules for immobilization in paper, Process Biochemistry, 46(6), 2011, P993-1000.

that significant alteration of the enzyme's conformation occurs in the presence of PEI, an effect of the cationic polymer that was not observed with GOx. Microencapsulation improved the thermal stability of GOx at temperatures up to 60°C due to stabilization of its active conformation but reduced the thermal stability of laccase because of the increased coordination between PEI and copper atoms in the enzyme's active site. Preliminary GOx bioactive paper was fabricated, which could be potentially used as food packaging paper.

Keywords

Bioactive paper, enzyme, conformation, microencapsulation, stability, immobilization

## 5.2 Introduction

Enzymatically-modified paper strips show great promises as inexpensive, portable and widely distributed biosensors. Several examples of bioactive papers were produced either through physical adsorption of the biorecognition elements [1] or by printing sol-gel-derived bioinks on the paper [2]. In our lab, bioactive paper is prepared by incorporating poly(ethyleneimine) microcapsules containing enzymes into paper pulp, which we demonstrated to be an efficient and scalable method to retain biomolecules in paper sheets [3]. Microencapsulation consists of the entrapment of a species within beads or shells often made by a polymer. Many strategies have been developed to encapsulate enzymes [4] that could potentially be of interest to immobilize enzymes on paper substrates. These techniques include layer-by-layer wall formation, ionotropic gelification, and interfacial polycondensation of a polymer [5-10]. The major challenge of microencapsulation

technique is to maintain the enzymatic activity of entrapped enzymes. Liu [11] reported that immobilized GOx in alginate/chitosan microspheres maintained around 90% of the original activity after lyophilization and storage at -20$^{o}$C for a month. However, encapsulated protein molecules can be released from the pores of Ca-alginate gel beads which appeared to collapse after lyophilization due to the highly porous gel network. In addition, high porosity of CaAlg gel beads also allows chitosan to diffuse deeply into the gel core and possibly causes desorption of GOx. Alginate/chitosan microspheres [11-13] have also been used to immobilize enzymes and since this technique does not involve any chemical crosslinking process, immobilized enzymes maintained high activity. Despite these advantages, the microspheres prepared by this method usually have a relatively large diameter and loaded protein can be released easily from the pores of gel beads.

PEI is often found in use in binding, immobilization and entrapment [14-23]. However, many authors reported a decrease of enzymatic activity in PEI-based immobilization systems. Matella *et al*. [24] for example studied the immobilized enzyme system using poly(ethyleneimine), glutaraldehyde (GA), and cotton cloth and reported an approximately 50% to 90% of enzyme inactivation with the combination of PEI and GA. The enzymatic activity losses after immobilization were attributed to unfavorable interactions between proteins and polyelectrolytes that could result from van der Waals forces, dipolar or hydrogen bonding, electrostatics forces, and hydrophobic dehydration [25, 26]. The conformational changes resulting from these interactions, together with the limited diffusion of enzymes immobilized in dense polymeric networks, may all be are held

responsible for the reduction of enzymatic activity during microencapsulation and determining the exact cause of enzymatic activity loss from the encapsulation processes is therefore a key step in the development of our immobilization platform based on microencapsulation.

Some strategies to immobilize biocatalysts on various supports using PEI are discussed in a review article from Bahulekar *et al.*[27] In a previous publication, [28] we have compared two simple and efficient enzyme microencapsulation methods based on interfacial polymerization of PEI and evaluated the microcapsules' properties by several characterization methods. The intent of the current paper is to investigate the enzymatic activity of free and encapsulated enzymes since enzyme activity loss occurred during the microencapsulation process by interfacial polycondensation of PEI has never been reported. Takashi *et al*. [29] used polyamide microcapsules prepared by interfacial polycondensation to entrap trypsin (I) and found that $K_M$ and $V_{max}$ values were considerably higher for free trypsin in solution than for immobilized trypsin. As far as the authors are aware of, there are only very limited reports on biological encapsulation using interfacial polymerization method, even when this method provides a tight control on microcapsules mean diameter in a fast preparation. In addition, no report on the specific interactions between microencapsulated enzymes and membranes of PEI microcapsules can be found in the literature. Here, PEI microcapsules were prepared by a microemulsion method and used to entrap two different enzymes (GOx and TvL), with a high entrapment efficiency. The activities of both enzymes were investigated throughout the various steps of

the encapsulation procedure to provide an explanation of the loss in enzymatic activity observed.

## 5.3 Materials and methods

### Materials

Polyethyleneimine (PEI, Mw = 1300 Da; 50 wt% soln. in water), sebacoyl chloride (SC), cyclohexane (CH), Span 85, bovine serum albumin (BSA), TvL (EC 1.10.3.2, ca. 10% protein content, 22.4 $U.mg^{-1}$), *p*-phenylenediamine (PPD), D-(+)-glucose, fluorescamine and QuantiPro BCA (bicinchoninic acid) Assay kit were purchased from Sigma-Aldrich. Other salts used for buffer preparation were purchased from A&C American Chemicals Ltd. Glucose oxidase from Aspergillus niger (GOx, E.C. 1.1.3.4, specific activity @25 $^{o}C$ >110 $U.mg^{-1}$) was obtained from Amersco, water was purified with a Milli-Q purification system to a specific resistance of at least 18 $M\Omega \cdot cm^{-1}$.

### Enzyme microcapsules

Enzyme microcapsules were prepared by a microemulsion technique adapted from that of Poncelet *et al.*[30] and described in details in a previous publication [28]. In short, various weights of enzymes (laccase: 1 to 50 mg, glucose oxidase: 5 to 60 mg) between 1 and 50 mg (see Table 5-1), were dissolved in a McIlvaine buffer (0.2 M $Na_2HPO_4$ and 0.1 M citric acid) solution (10 mL) containing PEI (4.6% v/v). This aqueous phase was emulsified in cyclohexane for 15 min prior to the addition of the cross-linking agent sebacoyl chloride. The microcapsules obtained were firstly rinsed with cyclohexane and

subsequently with Milli-Q water to remove unbound PEI molecules. PEI microcapsules can be prepared efficiently from the fast interfacial condensation of the polymer with a diacid chloride [28], and the reaction leading to membrane formation is shown in Figure 5-1.The loading of laccase in the microcapsules was calculated from the amount of laccase used in the preparation (see above) multiplied by an encapsulation efficiency factor of 0.94 which was determined evaluated earlier [28].

**Measurement of enzymatic activity**

The activities of TvL and GOx were determined from the rate of oxygen consumption by the enzymes in the presence of its substrate (*o*- or *p*-phenylenediamine (OPD or PPD) for TvL and glucose for GOx) rather than by conventional spectroscopic methods which provided unreliable absorbance readings for turbid suspensions of microcapsules. Oxygen consumption measurements of free laccase in solution were achieved by adding an aliquot of the enzyme solution in a buffer (total volume = 3 ml) in the chamber of an oxygen cell (Rank Brothers Ltd.) and by following the decrease in $O_2$% over time upon addition of the substrate to the cell.

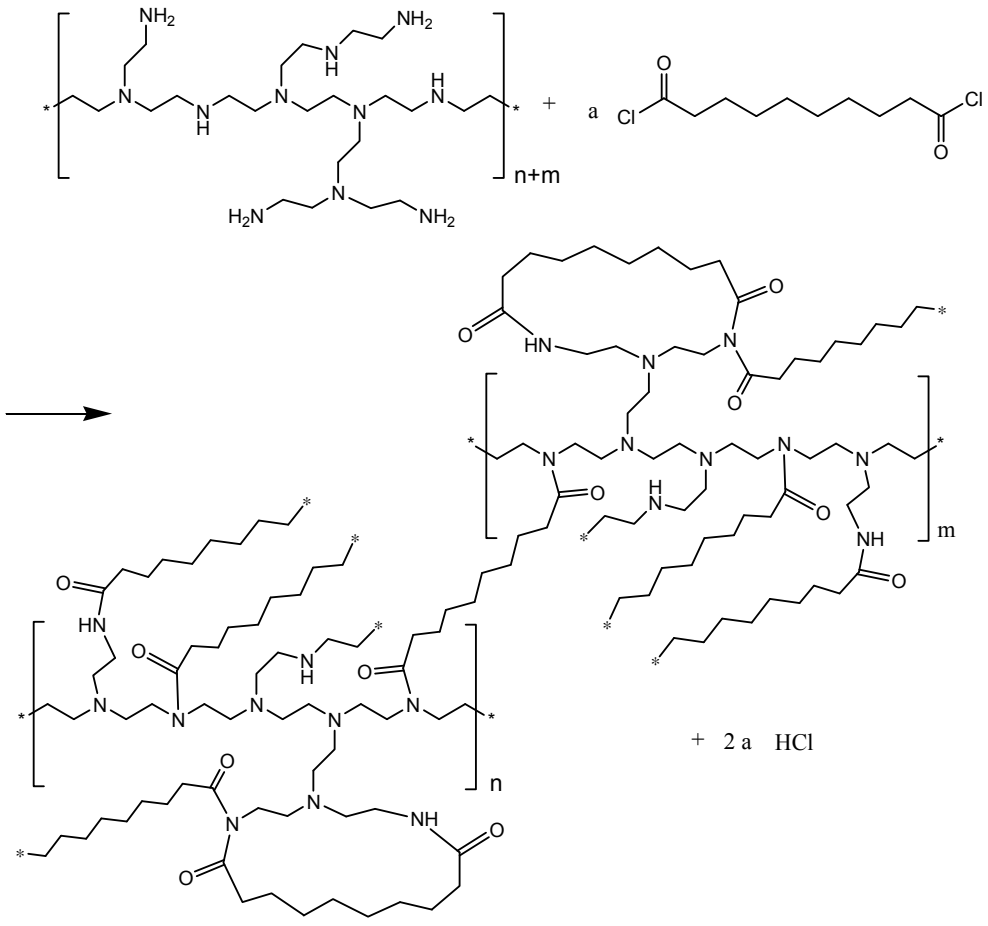


Figure 5- Scheme of the cross-linking reaction during encapsulation showing several possible linkages between PEI chains.

A similar procedure was followed with microcapsules except that a weighted amount of capsules was introduced in the cell chamber. To obtain a sufficient amount of enzyme for the measurement while maintaining substrate saturation conditions, the amount of microcapsules to be introduced in the cell was determined to provide a value of 0.1 U/ml (the amount was calculated using the initial laccase loading in the capsules (Table 5-1)). In all cases, the full scale of the instrument was calibrated prior to the measurement by

purging the buffer solution with $N_2$ (0%) and by saturating the solution with air (100%) afterwards. The measurement chamber of the instrument was kept at 25°C for all experiments using a water jacket and the initial rate of any given reaction was then obtained from the slope of the graph. The substrates selected for TvL activity measurements were *o*- or *p*-phenylenediamine (OPD or PPD) which are either neutral or slightly cationic in the pH range required to sustain activity.

**Fluorescence spectroscopy Assay**

A Perkin Elmer Luminescence spectrometer LS50-B was used to investigate the conformation of both TvL and GOx in the presence of PEI at room temperature. The excitation wavelength was set at 280 nm and the emission spectrum was recorded from 295 to 400 nm for both enzymes. Fluorescence measurements were carried out in 0.1 M acetate buffer at a pH of 5 in a quartz cuvette of 10 mm length path. All emission spectra presented were obtained by averaging 20 individual scans.

**Circular dichroism (CD)**

CD spectra of TvL and GOx in the absence and presence of PEI were recorded on a Chirascan (Applied Photophysics) instrument using a 500 μm light path cuvette at room temperature. Both enzymes were dissolved in a McIlvaine buffer at a protein concentration of 1 mg/mL. All spectra presented were corrected by subtracting from the baseline which was recorded with the buffer, for pure enzyme spectra, or with PEI dissolved in buffer solution for all other spectra.

Table 5- Decrease in laccase activity observed after the microencapsulation procedure. The activity was measured by the oxygen consumption method in the presence of 4.4 mM of substrate PPD in 50 mM McIlvaine buffer (pH 5) at 25°C.

| Enzyme | Condition | TvL amount loaded in microcapsules (mg) | Mass of microcapsules used for activity measurement (mg) | Total laccase amount in $O_2$ cell (µg) | Specific activity (%$O_2$/s·g of laccase) | Residual activity vs. free laccase (%) |
|---|---|---|---|---|---|---|
| TvL | Free (solution) | | - | 15 | 5.7 | 100 |
| | Encapsulated (suspension) | 1.0 | 405 | 20 | 1.8 | 32 |
| | | 5.8 | 74 | 20 | 2.1 | 37 |
| | | 9.9 | 45 | 21 | 1.9 | 33 |
| | | 25.9 | 16 | 19 | 1.8 | 32 |
| | | 49.7 | 10 | 23 | 1.5 | 26 |

## 5.4 Results

### 5.4.1 Enzymatic activity of microencapsulated enzymes

The effects of the microencapsulation procedure on the activities of TvL and GOx were evaluated by comparing the mass-specific activity of free and encapsulated enzymes using the $O_2$ consumption method described in measurement of enzymatic activity. Table 5-1 shows the comparison of the enzymatic activities (presented in %$O_2$ consumed in the 3 ml cell per second) between a solution of free laccase (15 µg) and suspensions of TvL microcapsules with each sample containing ca. 20 µg. To ensure that the mass transport of

$O_2$ to the enzyme across the walls and aggregates was not limiting, we incorporated different amounts of capsules that were prepared with various quantities of TvL. A proper comparison of the activities was achieved by adjusting the amounts of microcapsules used for the activity measurements to obtain a fixed mass of TvL in the $O_2$ cell. The average residual activity of TvL once encapsulated, calculated from the results presented in Table 5-1, was 33%. A similar value, 35%, was obtained for glucose oxidase.

Further assessment of the effect of microcapsules on the enzymes was achieved by kinetic measurements. We have already demonstrated that a decrease in $V_{max}$ and $K_M$ occurred in microencapsulated TvL using either *o*- or *p*-phenylenediamine substrates [3, 31]. What we have observed here with the enzyme GOx and using glucose as a substrate is a decrease in $V_{max}$ but a $K_M$ that remained virtually unchanged (see Figure 5-2) In Figure 5-2, the data points were fitted to a hyperbola function to estimate $V_{max}$ at infinite glucose concentration and to calculate $K_M$ at $V_{max}/2$. The microencapsulation of GOx resulted in a small variation of $K_M$, from 19 (free) to 14 mM (encapsulated). In comparison, the $K_M$ for TvL decreased by 84% upon encapsulation [3], suggesting that the PEI membrane affected the TvL's affinity for its substrate to a greater extent than it did for GOx.

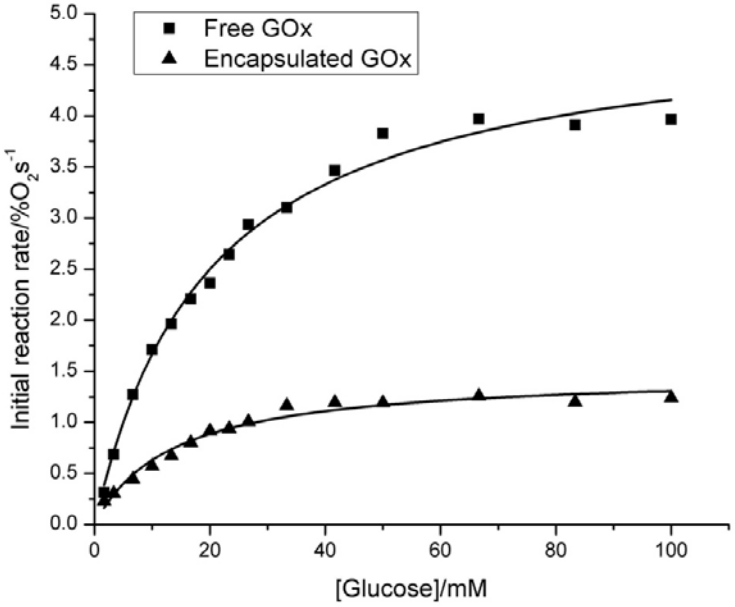


Figure 5- Michaelis-Menten behavior for the oxidation of glucose by free (squares) and microencapsulated (triangles) glucose oxidase. All initial rates were measured with an oxygen cell in a McIlvaine buffer at pH 5 and were expressed as the decrease in $O_2$ % content per second. 3.3 U GOx (107 mg microcapsules) in 3 ml of buffer solution was used for each measurement at 25°C; immobilized enzyme: 30.7 U GOx per gram microcapsules.

The thermal stabilities of TvL and GOx were studied by incubating a solution or a suspension of the free and encapsulated enzymes at 60°C for different periods of time. The samples were then rapidly cooled to room temperature and their activities measured in the $O_2$ cell at 25°C. Figure 5-3 presents the effect of the incubation at 60°C on the activities of GOx (A) and TvL (B). A drastic decrease of over 90% occurred for microencapsulated TvL

in less than 12 minutes. Oppositely, GOx presented a higher thermal stability when incorporated in microcapsules than the free enzymes, with the encapsulated GOx having a half-life time higher by a factor of 4 than that of free GOx. The inclusion of GOx in the membrane of the microcapsules could be responsible for this increased thermal stability by restricting the freedom of this large molecule (150 kDa) to undergo thermally-induced conformational changes or by preventing the dissociation of the enzyme subunits.

As described in Section 5.2.2, our microencapsulation procedure involves the use of several chemicals (cyclohexane, sebacoyl chloride, PEI and surfactants) that might affect the enzyme activities. To determine if any of these chemicals could be held responsible for the losses observed, the enzymatic activities of TvL and GOx were monitored along the different steps of the microencapsulation procedure. Table 5-2 presents the normalized activities (100% as free enzyme buffer at optimal conditions) of both enzymes measured immediately after each critical step of the procedure. PEI directly affects TvL's activity (a 78% decrease) while GOx's activity remained virtually the same. The interaction between TvL and PEI was hence investigated further.

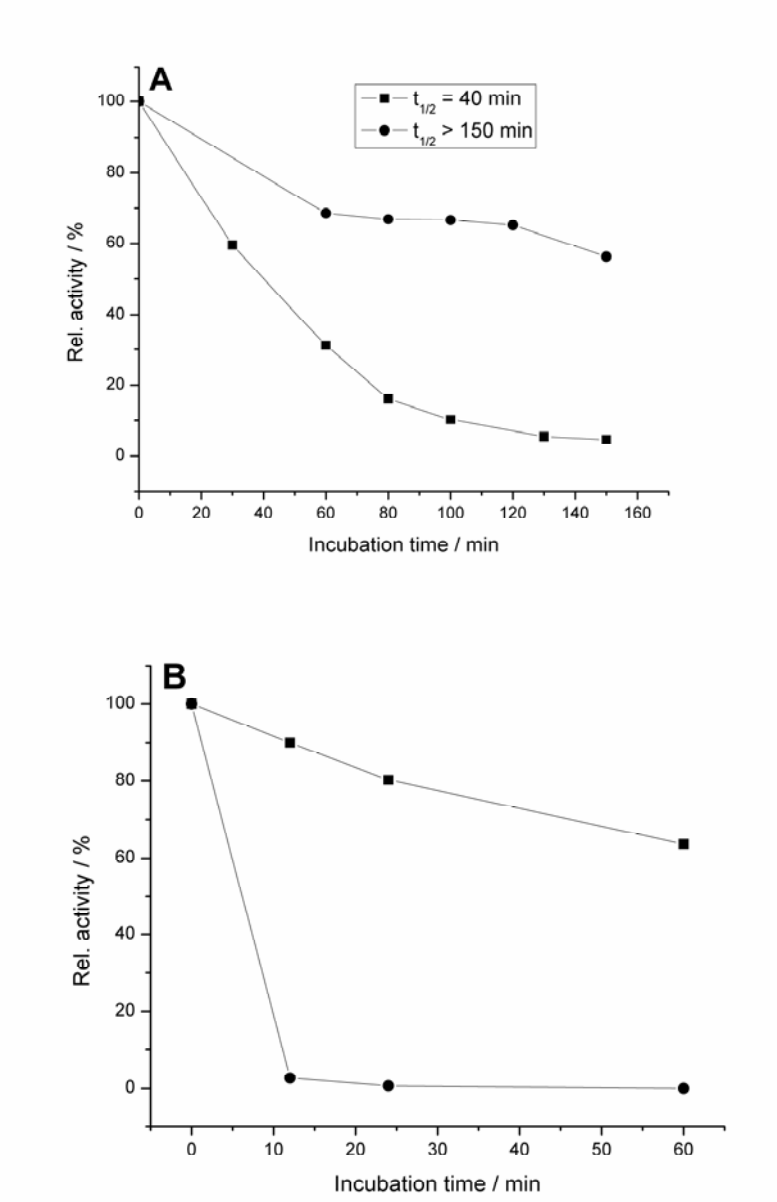


Figure 5- Heat inactivation curves obtained by measuring the activity of GOx (A) and TvL (B) after an incubation of a solution of the free (squares) and microencapsulated (circles) enzymes at 60°C. All solutions were cooled prior to activity measurement in the oxygen cell at 25°C.

Table 5- Monitoring of TvL's and GOx's activities by the oxygen consumption method, during the various steps of the microencapsulation procedure.

| Enzyme | Condition | Activity ($\%O_2/s$) | Relative activity (%) |
|---|---|---|---|
| TvL (0.012U/ml) | Buffer | 0.266 | 100 |
| | Buffer + 0.04 mM PEI | 0.059 | 22 |
| | Buffer, after emulsification in cyclohexane | 0.262 | 98 |
| GOx (5 U/ml) | Buffer | 0.578 | 100 |
| | Buffer + 0.04 mM PEI | 0.578 | 100 |
| | Buffer, after emulsification in cyclohexane | 0.575 | 99 |

To evaluate the activity inhibition on free, non-encapsulated TvL by PEI, kinetic measurements were carried out using *o*-phenylenediamine (OPD) as the substrate. In this case, TvL-catalyzed OPD oxidation was monitored over time with a UV-Vis spectrophotometer ($\lambda_{max}$ = 450 nm) in the presence of various concentrations of the suspected inhibitor PEI. Figure 5-4 shows the Lineweaver-Burk plots obtained for OPD oxidation with PEI concentrations ranging from 0 to 0.135 mM. The analysis of the curves, presented in the legend of Figure 5-4, shows a decrease in both $K_M$ and $V_{max}$, suggesting a noncompetitive inhibitory mechanism. These decreased values were also obtained for the encapsulated enzymes (see above and [3, 31]) although in the latter the reduced $V_{max}$ could be attributed to the hindered diffusion of the substrates across the microcapsules' membrane. The apparent noncompetitive inhibition suggested by the kinetic measurements

cannot however rule out the possibility of conformational changes occurring in the enzymes' structure in the presence of PEI. This possible cause of the decreased TvL's activity by PEI was therefore evaluated by fluorescence and circular dichroism measurements. Figure 5-5 shows the intrinsic fluorescence emission of tryptophan residues contained in the TvL's (A) and GOx's (B) structures. The emission scan of native TvL presents a maximal intensity at 350 nm. Upon addition of PEI, this peak decreased in intensity and shifted towards higher wavelengths due to the exposition of tryptophan residues to a different environment, suggesting a change in the enzyme's conformation. The fluorescent signal of GOx remained virtually unchanged in the presence of PEI, which is expected since GOx maintained its activity in the presence of PEI (Table 5-2). The photographs included as insets in Figure 5-5 were taken before (left vial) and after (right vial) the addition of PEI to the concentrated solutions of TvL or glucose oxidase. TvL solutions usually present a light green-blue color due to the Type 1 copper atom located in its active site. In the presence of PEI, the green-blue color turned to yellow, indicating a possible interaction of the polymer with either the active site's Cu or the amino acids in its vicinity. Additional information on the structural changes was gathered from circular dichroism experiments. Figure 5-6 represents the CD spectra of TvL (A) and GOx (B) with and without PEI. The band observed at the 220-240 nm of the spectra is attributed to the β-sheets arrangements in the enzymes' secondary structure. The 30% decrease in band intensity for the TvL enzyme clearly shows a disruption in the β-sheets induced by the polymer.

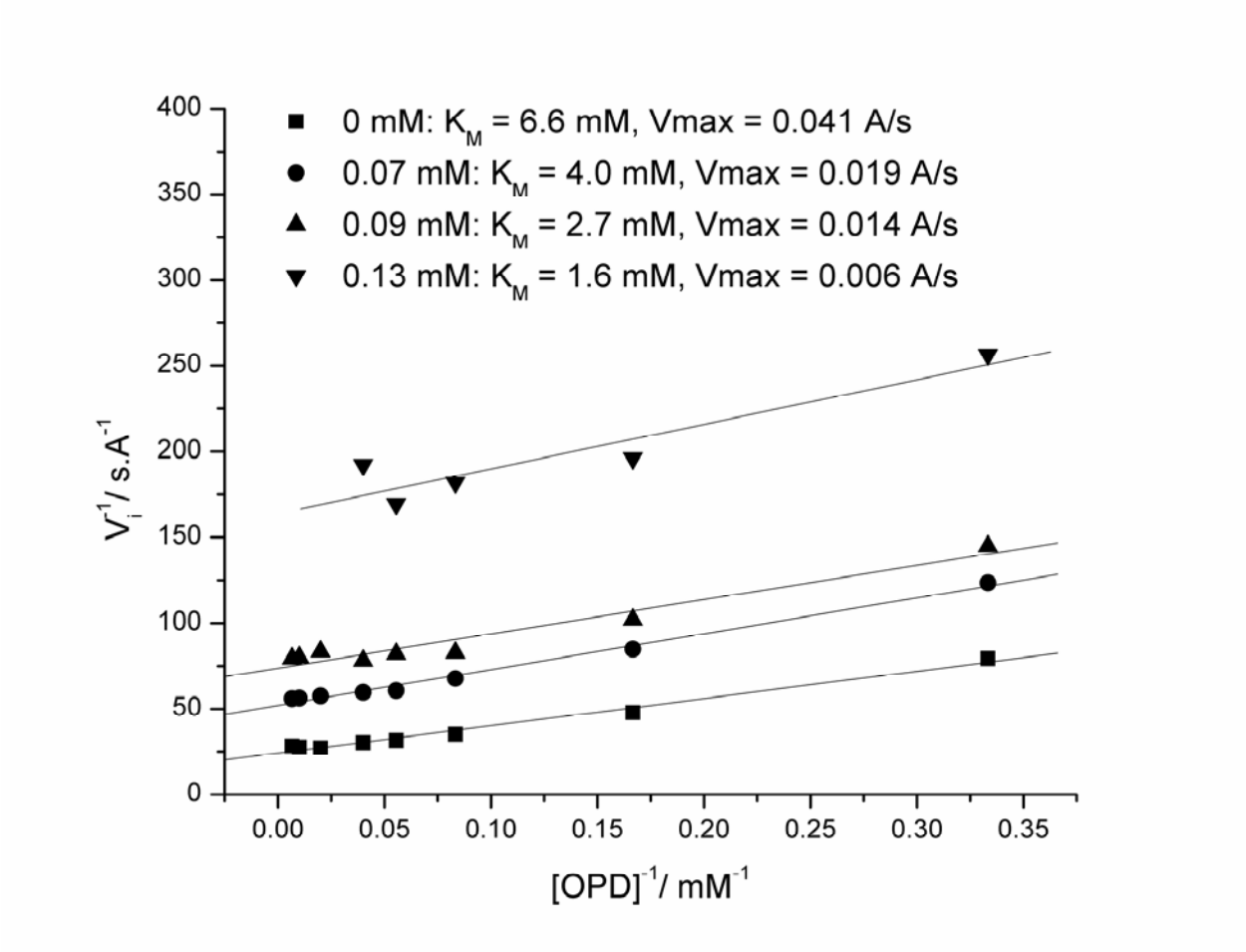


Figure 5- Lineweaver-Burk plot for the OPD oxidation by laccase in presence of PEI at different concentrations. Initial rates (Absorbance/s) for the reaction were measured at 450 nm in 50 mM McIlvaine buffer at pH 5 at 25°C.

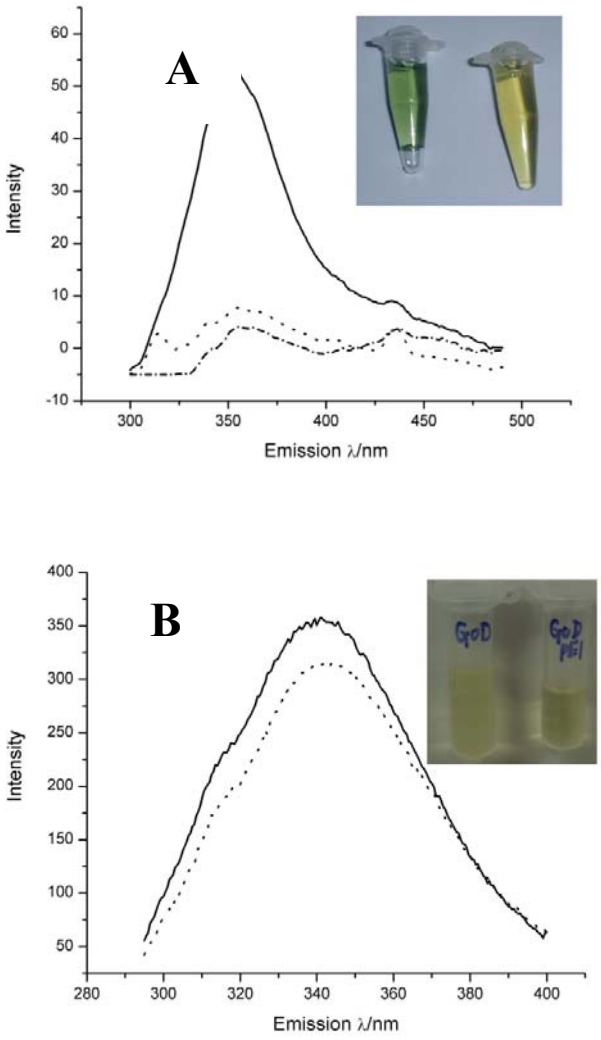


Figure 5- Intrinsic fluorescence emission spectrum of laccase (A) and glucose oxidase (B) in the presence (dotted line) and absence of PEI (solid line). The spectrum obtained for a solution of PEI in buffer is showed for comparison in A (dash-dot). Excitation wavelength was set to 280 nm in both experiments. The photographs in insets of the spectra were taken for a concentrated solution of the enzyme (left) and immediately after addition of PEI (right).

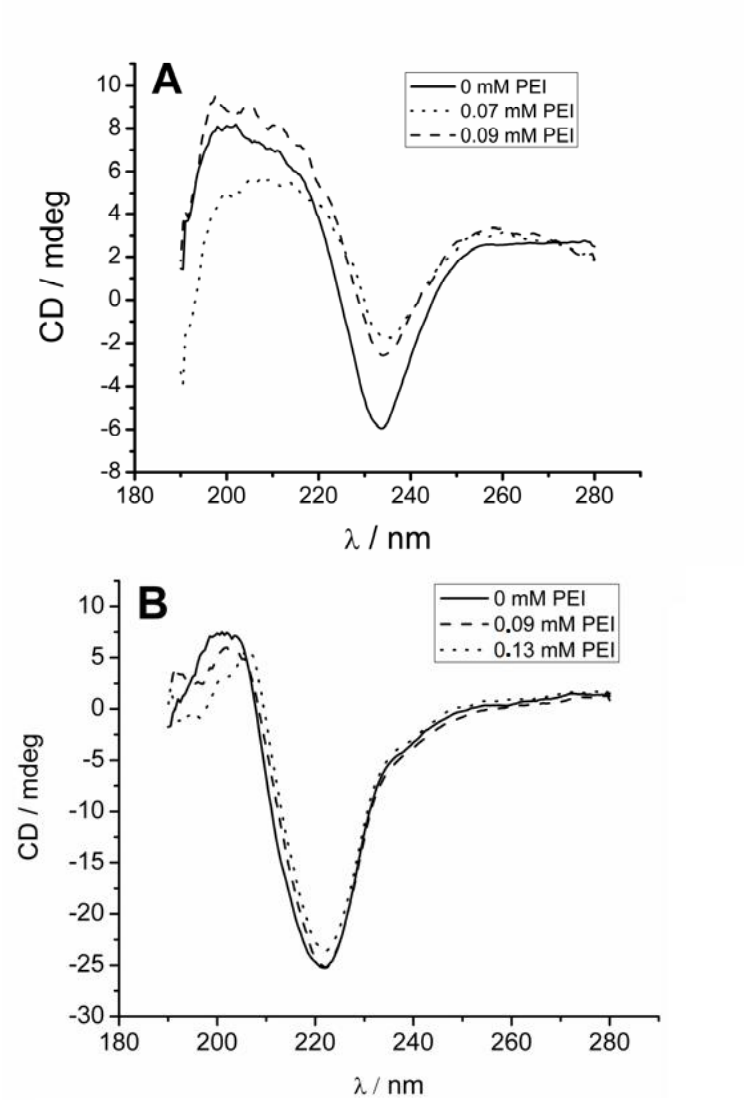


Figure 5- CD spectra showing the disruption of β-sheets arrangements in laccase (A) in presence of PEI. The spectra in B show no alterations for glucose oxidase by PEI.

### 5.4.2 Preliminary results on bioactive paper

The bioactive paper was fabricated by incorporating microcapsules of GOx in an aqueous suspension of bleached hardwood pulp. The paper sheets, in which the

microcapsules were uniformly distributed, were obtained by filtration of the suspension over a filter paper with a funnel and were allowed to dry at room temperature overnight prior to the measurements. Figure 5-7 (A) shows an image obtained with a wide field fluorescence microscope of a paper that was modified with GOx microcapsules. For the purpose of the measurement, the enzyme was fluorescently-labeled with fluorescamine prior to its encapsulation. Although some small aggregates can be found in the fibers, the distribution of microcapsules is relatively uniform. Figure 5-7 (B) shows the pictures of a paper strip modified with GOx microcapsules taken at different times after the addition of a solution of 0.5 M glucose and 0.5M KI. The color of the strip changed from yellow to dark brown due to the oxidation of $I^-$ to $I_2$ in presence of the $H_2O_2$ produced by the GOx-catalyzed oxidation of glucose in the presence of oxygen. Applying the reagents to a paper modified with microcapsules that does not contain any enzyme did not produce any color change (Figure 5-7 C), showing that encapsulated GOx is active in the paper and it is the enzyme that is responsible for the color change.

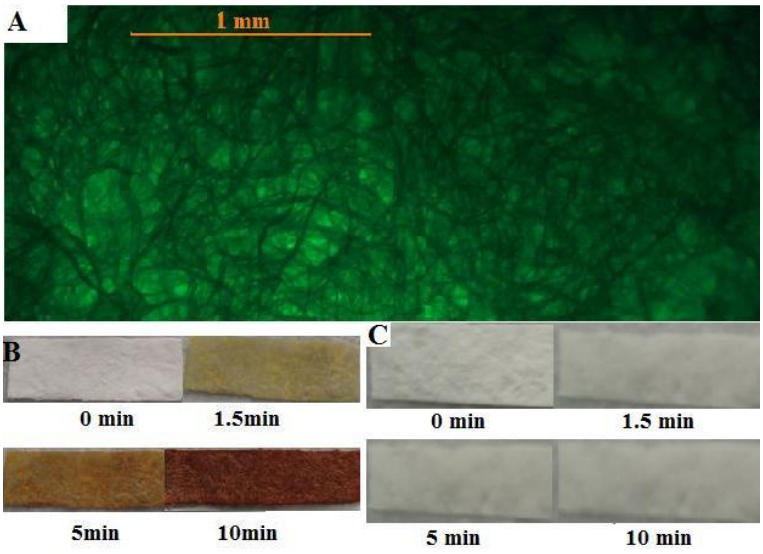


Figure 5- (A) Fluorescence micrograph of a 3 $mm^2$ section of a bioactive paper prepared by the incorporation of GOx-containing microcapsules in paper pulp suspension. The microcapsules were tagged by fluorescamine to obtain fluorescent signal. (B) and (C) are photographs of a paper prepared with GOx-microcapsules or non enzymatic microcapsules, respectively, and on which a solution containing 0.5 M glucose and 0.5 M KI was added.

## 5.5 Discussions

Microencapsulation of enzymes by interfacial reticulation of PEI resulted in the inclusion of the encapsulated proteins within the membrane of the capsules [32] as a result of the pH-dependent electrostatic interactions occurring between the polymer and the proteins. Incorporation of pH indicators in the aqueous phase during the encapsulation procedure revealed an initial pH of 11 (phenolphthalein) due to the basic nature of PEI. At pH 11, PEI is neutral but TvL and glucose oxidase are negatively charged (pIs of 4 and 4.5,

respectively). During the cross-linking reaction, sebacoyl chloride reacts by its end groups with the secondary and primary amino groups on the PEI Figure 5-1), releasing protons that decrease the pH of the aqueous phase to a final value between 6 and 8 (methyl red and phenolphthalein indicators). At neutral pH, PEI bears a positive charge (15-20% of amino groups are charged) [33] causing the negatively-charged proteins to interact with the polymer. Once the enzymes are brought in contact with the membrane in formation, cross-linking reaction can occur between the proteins' amino groups and those on PEI by the acid moieties at the ends of sebacoyl chloride, resulting in their irreversible immobilization within the membrane.

Inclusion of TvL and GOx in PEI microcapsules' membrane induces changes in their reactivity as it was pointed out by the reaction kinetic measurements. The maximum rate ($V_{max}$) of free enzymes was much higher than that of encapsulated ones, since the membrane of microcapsules reduced the substrate diffusion rate, some of enzyme molecules were crosslinked into membranes, and hydrophilic character of membranes of microcapsules can reduce the oxygen concentration inside of microcapsules. Diffusion of substrate into, and product out of, the capsules is achieved by the concentration gradient. The decrease in activity ($V_{max, app}$) may be explained by a rapid depletion of substrate in the environment immediately surrounding the enzyme, which is confined within the pores of the capsules. Since there is some diffusion restriction, the apparent activity would appear to be lower than it really is at the active site of the enzyme, since a macroscopic measurement is made in the bulk solution. A significant decrease in apparent Michaelis constant was

observed for TvL (400 to 65 mM, PPD oxidation [3]) while that of GOx remained the same (19 to 14 mM, glucose oxidation). An increase in enzyme-substrate affinity, suggested by the lower $K_M$, upon the inclusion of TvL in the membrane is possible and enzymes have been reported with lower $K_{M,app}$ in polymer films [34] and microcapsules [35]. Results from fluorescence and CD measurements pointed out that PEI affects laccase closely to its active site which can be used to explain the decrease in $K_M$ value for encapsulated laccase. GOx however does not see its structure modified by PEI and $K_M$ stays unchanged upon encapsulation. Therefore, we conclude that, for both enzymes, $V_{max}$ decreases in microcapsules due to diffusion restriction and substrate depletion. Laccase, which is most sensitive to PEI, has its active site altered in the presence of the polymer resulting in a better access of the substrate, hence a lower $K_M$ value (not observed with GOx). This difference provides an indication that the interactions between the enzyme and the polymer, and therefore the explanation of the activity losses, might be dependent on the nature of the enzyme rather than simply due to their inclusion in a polymeric membrane. Another indication is provided by the very different results obtained from heat inactivation measurements of both enzymes. We have observed an increase in the thermal stability of microencapsulated GOx that can be explained by the stabilization of the enzyme's structure within the membrane since cross-linking between the polymer and GOx must have occurred during the capsule membrane formation. An increased heat-resistance for GOx in polyurea microcapsules prepared by interfacial polymerization has previously been reported by Komori *et al.*[36] Conversely, microencapsulated TvL was more prone to heat inactivation than free TvL (Figure 5-3). This opposite behavior can only be explained by

the existence of detrimental interactions occurring between the PEI membrane and the TvL. In order to minimize these interactions, lyophilized microcapsules were subjected to the same heat treatment at 60°C. In the absence of water, microencapsulated TvL maintained its activity and no losses were observed within the first 15 minutes of incubation (vs. 90% activity decrease in solution). Under dry conditions, the polymer and the enzyme were not able to interact.

We therefore identified three possible explanations of the severe reduction in enzymatic activity of PEI encapsulated enzymes and that might help rationalize the lower $K_M$ observed or the differences in heat inactivation: protein losses, enzyme inhibition by reagents, and conformational changes. We have showed in a previous publication [28] that high encapsulation efficiency (96%) was achieved with the procedure, ruling out the possibility of the loss of large quantities of enzyme to the organic phase or during the washing steps of the preparation. Inhibition of TvL by PEI, on the other hand, was clearly demonstrated in Figure 5-4 where the LB curves suggested an uncompetitive inhibition behavior. The fluorescence and circular dichroism experiments determined that this apparent inhibition was in fact due to conformational changes in the TvL's structure. The chemical composition of PEI contains 16% nitrogen, of which 20% are primary amines, which are known to be able to coordinate with proteins [37]. If such interactions lead inevitably to a disruption of the enzyme structure, the changes observed with TvL would be expected to occur also in glucose oxidase. As showed above, the addition of PEI did not exert significant effects on GOx's conformation and the enzyme maintained its activity in

the presence of the polymer. It is noteworthy to mention that some researchers [38, 39] reported that higher amounts of PEI reduced the stability of GOx. To explain this discrepancy between the behaviors of TvL and GOx, we turned towards the possible complexion of the copper atoms from the TvL's active site and PEI. A sufficiently concentrated TvL solution would present a light green-blue color due to light absorption by the Type 1 copper at around 600nm (see inset of Figure 5-5). The rapid change to a yellow color with the presence of PEI was a strong indication that PEI interacted directly with the active site. PEI molecules are strong copper-chelating agent because of their secondary and tertiary amino groups [40], thus they could break or distort some ligands connected with coppers in the TvL's active site. In addition, the ion pairing between oppositely charged molecules could also exert a modest influence on the enzyme's stability [26]. PEI might also cause distortions of optimum bond lengths and bond angles with a tightly packed interior of enzyme which are determinant for the catalytic activity. This effect of PEI is demonstrated by its ability to inactivate complex biological agents. An N-acetylated version of PEI was reported by Haldar *et al*. as an efficient antiviral and antimicrobial agent [41]. The authors proposed that the virucidal action originates from the ability of PEI to interact with proteins and thus achieve in membrane disruption. We do not believe that such interactions between PEI and protein can be held responsible for the loss in the activity of encapsulated TvL since no modification to GOx's structure took place in the presence of PEI. In addition to our observations, Bolivar *et al*. reported on the successful use of PEI to stabilize the quaternary structure of the enzyme glutamate dehydrogenase [42], The authors coated the enzyme with PEI which was afterwards cross-linked with

glutaraldehyde to obtain a composite that exhibited significantly higher thermal stability in solution than that of the unmodified enzyme. We have also observed an increased thermal stability for GOx when incorporated in the membrane of PEI microcapsules. We therefore conclude that the metal ion chelating ability of PEI, rather than unfavorable interactions with the enzyme peptidic backbone, was responsible for the loss in TvL's activity. Such chelating events caused a disruption of the β-sheets in the vicinity of the TvL's active site and occurred in order to accommodate the large polymer. This disruption was clearly demonstrated by circular dichroism spectroscopy and was amplified at higher temperatures. A strategy based on template-assisted layer-by-layer microencapsulation is currently undergoing in our lab as a mean to prevent TvL from interacting with the PEI membrane with the goal of decreasing the loss in activity.

Since the physical incorporation of GOx microcapsules into paper pulp did not affect its enzymatic activity, GOx paper strips maintain the same activity as GOx microcapsules. As discussed above, the reduction of enzymatic activity of microencapsulated GOx is mainly due to the diffusion restriction, some of GOx molecules crosslinked in the membranes can also reduce its activity, thus microencapsulation only reduces its reaction speed with its substrate. GOx paper can potentially be used as food packaging products to improve shelf life by scavenging oxygen that might penetrate the physical barrier.

## 5.6 Conclusions

The current work aimed at comparing the enzymatic activity of free and microencapsulated TvL and glucose oxidase to determine the factors causing the loss of

enzymatic activity during the interfacial polycondensation of PEI. The reduction of enzymatic activity after microencapsulation was explained by multiple factors. In general, diffusion through these closed shells with pore size <5 nm is a slower process compared with free enzymes in solution. For encapsulated GOx, the diffusion limitation is the key factor that led to the reduction of enzymatic activity since no interactions between GOx and PEI were observed. Some other factors, such as reduction of the amount of oxygen inside microcapsules (substrate depletion) can also reduce it activity. PEI, however, affected the conformation of TvL and decreased its enzymatic activity. Microencapsulation improved thermal stability of GOx, but decreased the thermal stability of TvL, since the coordination between the copper center in TvL and nitrogen atoms in PEI molecules was increased at higher temperatures. Preliminary colorimetric bioactive paper strips were prepared by incorporating microencapsulated enzymes into the paper pulp. Depending on the properties of microcapsules, enzyme microcapsules can be immobilized in paper by different ways including coating, sizing and printing. Although the present works only focused on TvL and GOx, we believe that a similar approach is applicable to other enzymes, and it could be used to develop different functional and valuable bioactive paper.

## Acknowledgments

The authors acknowledge funding from the Natural Sciences and Engineering Research Council of Canada (NSERC) through the SENTINEL Canadian Network for the Development and Use of Bioactive Paper (Research Network Grant). We also thank

professors J. Pelletier and A. Schmitzer for useful discussions and access to instrumentation.

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# Chapter 6 Fast and effective bioactive paper for self-diagnosis of bacterial vaginosis[6]

## 6.1 Abstract

In this article, sensitive colorimetric bioactive paper was fabricated to determine sialidase–related diseases like bacterial vaginosis (BV) in a one-step and dry format spot assay with fast response and good storage stability. The paper was prepared by three simple steps. The first step involves preparation of poly(ethyleneimine) (PEI) microcapsules, the second step is to incubate positively charged microcapsules in negatively charged 5-bromo-4-chloro-3-indolyl-a-D-*N*-acetylneuraminic acid (BCIN) solution, an color enhancer nitro blue tetrazolium (NBT), and in the third step, paper was fabricated by incorporating incubated microcapsules into paper pulp. This paper changes color from white to dark purple in the presence of sialidase in as little as 6 minutes, and color could be enhanced with increased length of reaction time. In this reaction system, BCIN was the substrate for sialidase, NBT was the color enhancer, and PEI microcapsules acted as catalyst. The loading efficiency of BCIN was about 22.2%, and filtered BCIN solution could be reused for the next fabrication.

Keywords

Neuraminidase; Microcapsules; Bioactive Paper strip; Bioactive paper; Colorimetric biosensor

[6] This Chapter will be submitted to Lab on a chip as a paper.

## 6.2 Introduction

Bacteria vaginosis (BV) is the most common vaginal infection in women of child-bearing age. Normal vaginal flora consisting of hydrogen peroxide-producing lactobacilli are replaced by *Gardnerella vaginalis*, *Mycoplasma hominis*, *Mobiluncus* species, which are anaerobic gram-negative rods in women with BV [1]. BV is strongly associated with gynecologic and obstetric complications including an increased risk for salpingitis, endometritis, pelvic inflammatory disease (PID), chorioamnionitis, premature rupture of membrane, and pre-term birth. BV is also associated with higher susceptibility to sexually transmitted pathogens, including HIV [2]. A number of studies have shown BV to be associated with elevated sialidase (also known as neuraminidase) activity, which is an abnormal condition of the vaginal ecosystem caused by a flora shift from the *lactobacillus* species present in normal conditions to overgrowth of a variety of aerobic and anaerobic vaginal bacteria, including *Gardnerella vaginalis, Bacteroides, Prevotella, Mobilincus* and *Mycoplasma* species [3].

Many kinds of BV tests exist, The Amsel criteria [1, 4-9] are the gold standard currently, which require the presence of at least three of four clinical signs set forth by Amsel et al: (i) Presence of abnormal vaginal discharge; (ii) Elevated vaginal pH (>4.5); (iii) Positive amine odor; (iv) Presence of clue cells on Gram stain or saline preparation of vaginal secretions. The Nugent Gram stain criteria [5, 7, 10-12] measure specific gram-stained bacterial morphotypes. This method is to use a Gram stained

evaluation of vaginal smears with the Nugent criteria or score [12]: colorimetric pH test: 72% and 67%; Papanicolaou smear: 72% and 79%. [13] Consequently, organisms that do not Gram stain, such as ureaplasmas and mycoplasmas, are not measured [7]. Some other detection methods, such as the Whiff test [7, 14-17], and the Ison and Hay's criteria, [18] were also developed. However, these conventional methods for diagnosing BV rely on an expert assessment and laboratory tests. The Nugent and Amsel criteria are considered as the most effective assessment for BV, but they are slow and accordingly dependent on specialist interpretation and laboratory equipment [19].

There has long been a need for a rapid, uncomplicated diagnostic test for BV. Since high levels of sialidase activity have been found in women with BV, colorimetric detection based on the detection of sialidase is becoming a promising method. But only the BVBlue assay [19-22] has been commercialized, it has a fast response (approx. 10 min), but it is not portable since it is a two-step liquid-phase chromogenic solution test kit. The active component for BVBlue assay is IBX-4041, which refers to the invention of US patent 2003/0162240 A1. The color reaction includes two steps. The optimal pH for the first hydrolysis step is 5.5-6, and the second color development step requires pH of ≥9. It is based on detection of the enzyme sialidase produced by pathogenic vaginal bacteria, and should be performed in a physician's office or a lab. This means patients cannot self-test at home. Since 2000, BCIN has been used as the substrate of sialidase for the detection of BV by Wiggins. [23] This method was first developed by some other authors, such as Reukov et al. [24] who immobilized BCIN onto nanocoated

fibers. The portable biosensor shows very slow response, takes at least 2 h to light up, and BCIN loading efficiency is only about 18%. The reactions for all of above assays were shown in Eq. 6-1 A. Sialidase in specimen catalyzed hydrolysis reaction of BCIN and produced light yellow color indoxyl isomers at pH 5.5-6, produced isomers were oxidized at higher pH 9 to generate visible blue color.

The method was described in US Patent 7591978 B2 [2, 3] is a dry format assay. Same substrate BCIN was assembled onto a sheet device with color enhancer of nitro blue tetrazolium (NBT) and some other compounds, while it gives response within a reasonable time, the device assembly method and detection step are complicated and expensive. Innovation of this method is the addition of color enhancer NBT, which decreases total reaction time. The reactions were illustrated in Eq. 6-1 B. First step is the same as previous publications such as Wiggin's and Reukov's, hydrolysis reaction of BCIN catalyzes by sialidase at pH 5.5-6 to produce indoxyl isomers; indoxyl isomers are oxidized in the presence of NBT at pH 9 to generate dark purple precipitate. It can detect liquid flow as a lateral flow assay and running buffer solution is necessary for the sample preparation. Moreover, the assembly method is not compatible with industrial fabrication. Furthermore, storage stability, which is a very important factor in potential applications, was not discussed.

Here, we report a method of bioactive paper fabrication method for the detection of sialidase, which could be applied to BV diagnosis by women. PEI microcapsules soaked in BCIN and NBT solution were incorporated into paper pulp for the fabrication

of bioactive paper. The paper can be used for the detection of sialidase-related diseases like BV. This kind of BV paper strips were resulted efficient in permitting to obtain a fast evaluation of the risk of pathologies related to the presence of sialidase with good storage stability. There is no sample preparation step and detection is as fast as 6 min, suspected patients can test by themselves before visiting a physician' office. Such bioactive paper strip was expected to be used not only by women on a day-to-day basis, but also by a physician as a fast determination.

## 6.3 Materials and methods

### Materials

BCIN was purchased from Toronto Research Chemicals Inc. NBT, PEI (50 wt% soln. in water, MW: 1300), sebacoyl chloride, cyclohexane, Span 85, and neuraminidase from *Clostridium perfringens* (C. Welchii, TypeV) were purchased from Sigma-Aldrich. Salts used for buffer preparation were purchased from A&C American Chemicals Ltd and MilliQ water (18 MΩ·cm) was used in the preparation of all solutions.

#### 6.3.1 Preparation of microcapsules

PEI microcapsules were prepared by the mechanical method via interfacial polycondensation. The detailed method has been described by our group [25]. Briefly

hydrophilic microdroplets of McIlvaine buffered PEI and Span 85 in cyclohexane (continuous phase) were crosslinked by sebacoyl chloride to form microcapsules.

### 6.3.2 Fabrication of bioactive paper

Neuraminidase (Sialidase, acetyl-neuraminyl hydrolase) can catalyze the hydrolysis of α2-3, α2-6, and α2-8 linked N-acetyl-neuraminic acid residues from glycol-proteins and oligosaccharides. BCIN is a known chromogenic substrate of neuraminidase [23], but the two-step color reaction requires different optimal pH. So it is not possible to make a one-step assay. Tetrazolium salts are known as highly sensitive color indicators in combination with 5-bromo-4 chloro-3-indolyl-phosphate for the detection of nucleic acid, protein, or glycoconjugates and are very common oxidizers in staining applications [26-29]. NBT was used here to oxidize the intermediate (halogenated indoxyl) of sialidase-catalyzed hydrolysis of BCIN and form dark purple product [3]. Then neuraminidase substrate was comprised by BCIN, NBT and PEI microcapsules for the fabrication of bioactive paper.

Four kinds of paper were fabricated with paper pulp. The materials used for their fabrication are listed in Table 6-1. A 0.01 M pH 6 phosphate buffer was used in paper fabrication. Samples were obtained in sheet at a ca. 95% consistency and without any retention agent used. Prior to handsheet formation, hard wood kraft pulp were suspended in water or buffer using a blade mixer for 16 h. Mixed suspensions were transferred to the top of Whatman No. 4 filter paper with pore size 20-25 μm in a

Buchner filter funnel with diameter of 7 cm. In papers 1 and 2, microcapsules were soaked in BCIN and NBT solutions for 30 min, and then mixed with paper pulp. Microcapsules only were socked in BCIN solution for the preparation of paper 3. Paper 4 did not contain microcapsules; only the paper pulp was mixed with BCIN and NBT solutions. In all 4 cases, the microcapsules and paper pulp used for paper preparation were wet. The paper after filtration was dried by purging with nitrogen gas. Since filter solutions of papers 1-4 contained enough BCIN and NBT, which can be reused to soak new batch of microcapsules to fabricate new paper strips.

Table 6- Materials used for the preparation of four kinds of paper

| Paper | Pulp /g | Microcapsules / g | BCIN/ $\times 10^{-7}$ mole | NBT/ $\times 10^{-7}$ mole |
|---|---|---|---|---|
| 1 | 1 | 1 | 3.1 | 7.1 |
| 2 | 1 | 1 | 3.1 | 3.6 |
| 3 | 1 | 1 | 3.1 | 0 |
| 4 | 1 | 0 | 3.1 | 7.1 |
| 5 | 1 | 0 | in $6.88\times10^{-8}$ mole BCIN + $1.58\times10^{-7}$ mole NBT | |

In order to visualize the function of PEI microcapsules, paper 5 was fabricated. Paper pulp was suspended in pH 6 buffer solution and the paper was prepared by filtration. Wet paper was dried by purging with nitrogen. This pure paper was soaked in BCIN and NBT solutions and dried again by nitrogen gas. Paper 5 contained the same amount of BCIN and NBT as calculated from paper 1(see Tables 6-1and 6-2).

The reaction taking place on the papers 1 and 2 required two steps. The first step was hydrolysis of BCIN catalyzed by sialidase in the specimen, and the second step is

the oxidation of indoxyl isomers from hydrolysis reaction with redox indicator NBT in the presence of PEI microcapsules formed a dark purple colored products. Secondary amine groups on PEI membranes acted as a catalyst in the second step of the reaction (Eq. 6-1 C). Unlike the description in previous publications, this both steps could be performed at the same pH value.

For the determination of paper activity, a very small amount (2-20 μl) of diluted neuraminidase was directly added to small piece of paper, and then paper strips were scanned at different times (from time 0 to 36 h) and mean grey values of scanned images were taken by Photoshop. Mean color intensities of different images were calculated by mean grey values (Mean color intensity = 256-mean grey value).

Neuraminidase was dissolved in 0.01M pH 6 phosphate buffer, since the optimal pH for its catalyzed hydrolysis reaction is pH 5.5-6. Normally, the vagina is slightly acidic with pH of 3.8-4.2, but it was elevated for the patients with bacterial vaginosis, so the elevated vaginal flora is close to this pH value.

### 6.3.3 Loading efficiency of BCIN

BCIN loading efficiency was evaluated using a UV-Vis spectrophotometer. Since BCIN has high UV absorbance at 280 nm, a standard curve was made by plotting assay data to determine the concentration of BCIN. The assay was performed with various known concentrations of BCIN by plotting concentration on the X axis and absorbance on the Y axis. And then the absorbance of filter solution for bioactive paper preparation was measured by UV-Vis spectrophotometer. The loading efficiency was obtained from

comparison of BCIN concentration in the filter solution and original solution before paper preparation.

## 6.4 Results and discussion

### 6.4.1 Microcapsules in bioactive paper

PEI microcapsules [25] for the entrapment of large enzyme molecules were used here to soak up small molecules for the preparation of sialidase-sensitive paper. Photos of the microcapsules are shown in Figure 6-1(a) (wet microcapsules viewed with a optical microscope) and Figure 6-1(b) (dried microcapsules using a scanning electron microscope (SEM)). Dried microcapsules could be rehydrated many times. Figure 6-1(c) represents fabricated paper containing microcapsules, in which microcapsules and their aggregrates were fixed in the kraft hard wood paper pulp.

### 6.4.2 Different kinds of paper

Since PEI-SC microcapsules are positively charged and BCIN is negatively charged at pH 6, BCIN can absorb onto the surface of microcapsules due to the electrostatic interaction. Membranes of microcapsules are porous, which allows absorption of small molecules like BCIN. In addition, the pore size of the membranes allows the diffusion of small molecules [25], thus BCIN and NBT molecules can diffuse across the membranes and remained in the core of microcapsules and absorbed onto outer membranes of microcapsules due to electrostatic interaction. Microcapsules with mean

size of 20 μm and their aggregrates can be fixed into paper with pore size ranging from 0.01-10 μm. Therefore, papers 1 and 2 can retain BCIN and NBT molecules due to existence of PEI microcapsules. On the other hand, both paper pulp and BCIN are negatively charged at pH 6 and the size of BCIN and NBT molecules are much smaller than the pore size of paper (0.01-10 μm). Therefore, paper 4 without microcapsules lost its BCIN and NBT after filtration, and thus it did not show any color response to neuraminidase (Fig. 6-2).

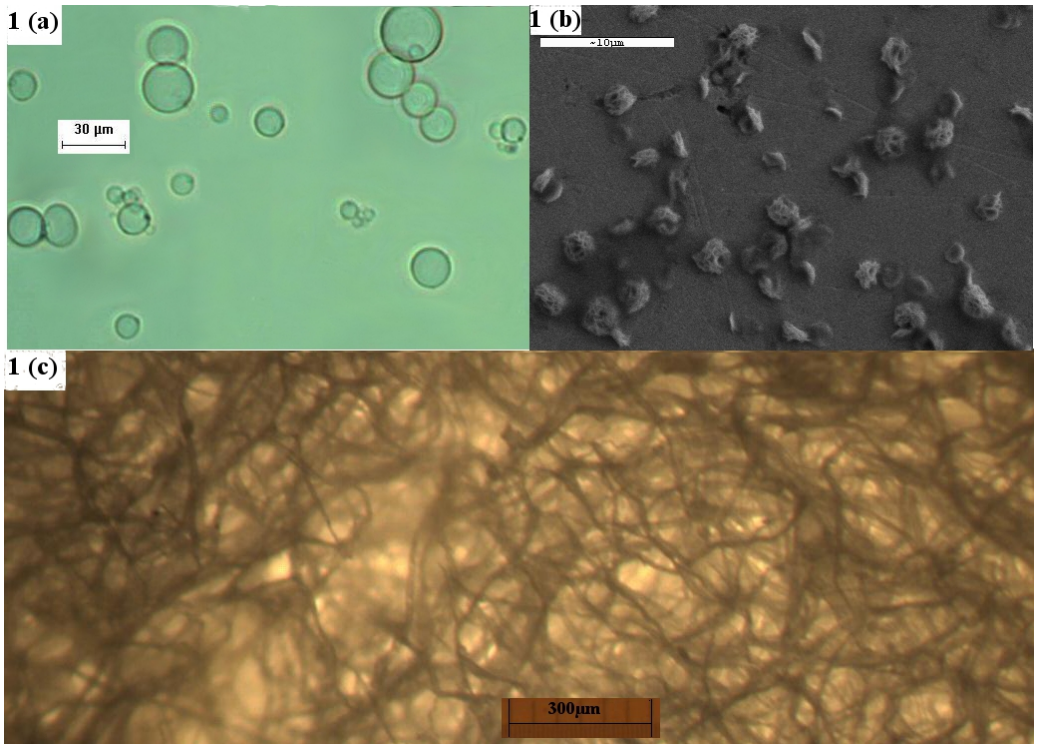



Figure 6- Microcapsules in wet (1(a)) and dried (1(b)) format and microcapsules in paper (1(c)). The wet microcapsules (1(a)) and microcapsules in paper (1(c)) were obtained from optical microscope; the dried format micrograph was obtained by SEM, and copper was used as the support.

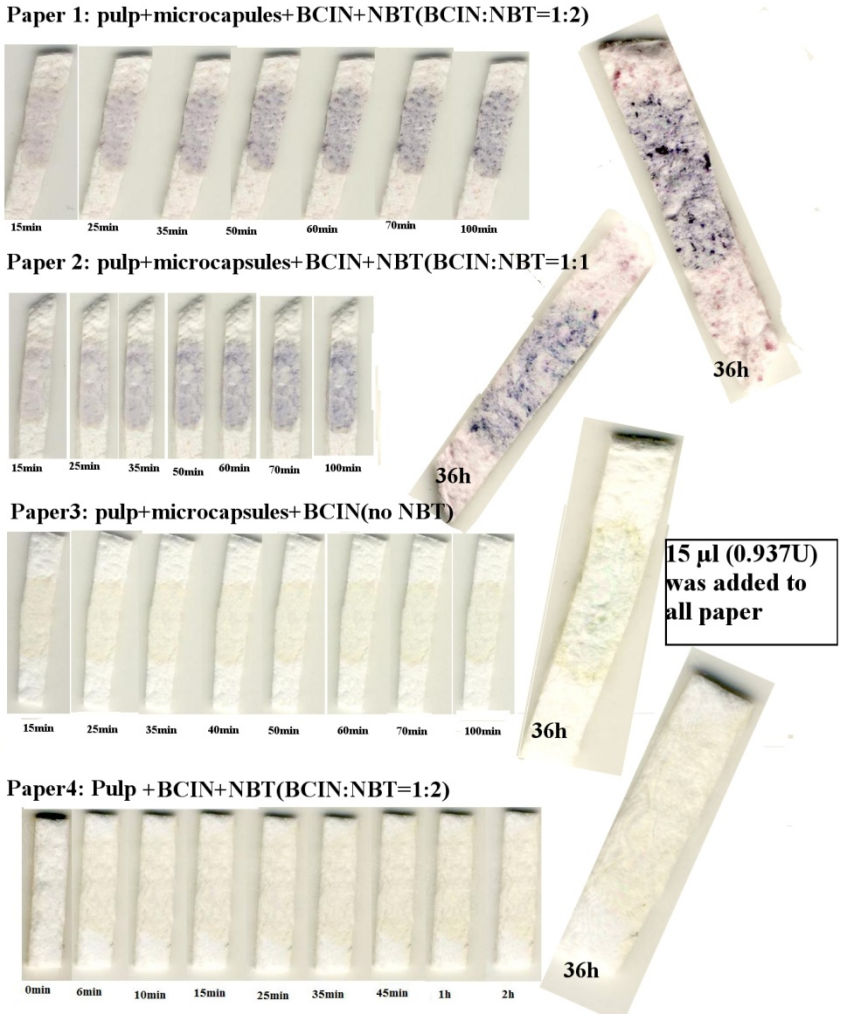
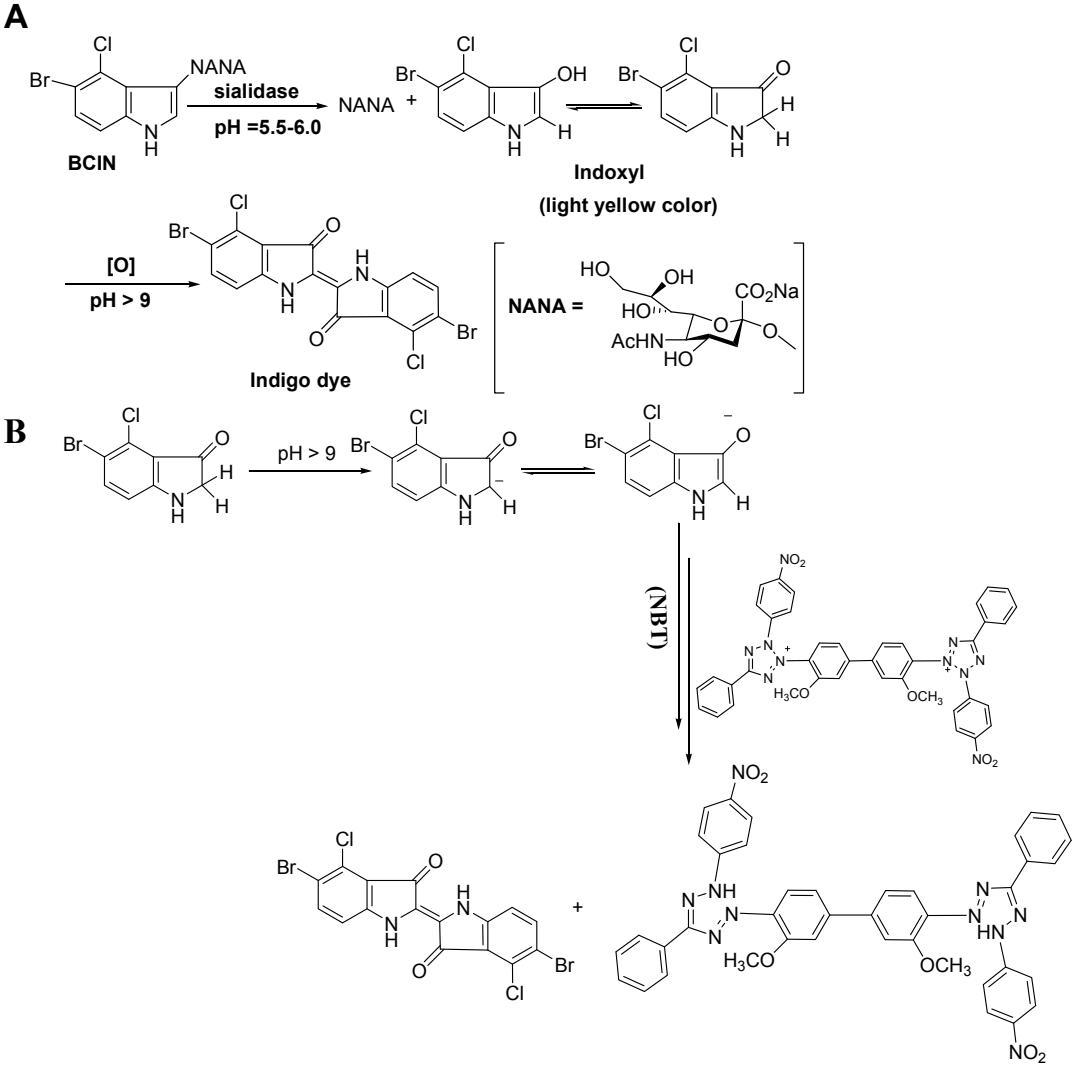


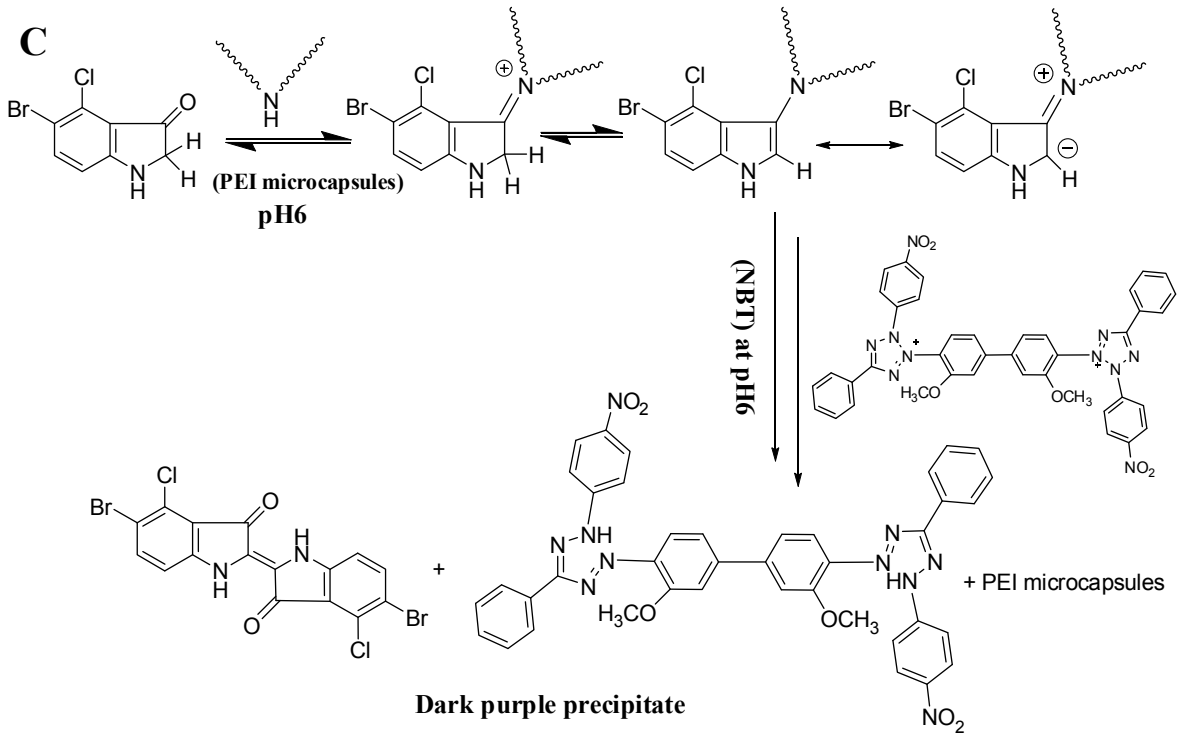

Figure 6-. The color reaction of four kinds of paper in the presence of sialidase. Paper 1 and 2 gave fast and significant color response in the presence of sialidase, which indicates the hydrolysis of BCIN and oxidation reaction of the intermediate accelerated by alkalization of NBT. Paper 3 showed a little bit of blue color after 2 hours, and was easy to recognize the color at 36 hours, which indicated the hydrolysis of BCIN in the presence of sialidase at pH 6. Paper 4 did not give any color response, which indicated there was no BCIN and NBT remaining in the paper.

Microcapsules were used for the fabrication of paper 3, so the substrate BCIN remained in it. The reaction mechanism is shown in Equation 6-1A. Since this is a one-step spot assay, only sialidase solution in pH 6 buffer was added to the paper strips. The second reaction step in Eq. 6-1A was very slow. The color changes of paper 3 after adding neuraminidase are shown in Figure 6-2, where the paper did not give a visible color within 100 min; only the photo at 36 h showed a little bit of green color. Since the second color development step requires higher pH and the intermediate (halogenated indoxyl) from the first hydrolysis step took a longer time to be oxidized at pH 6, so the color response is very slow. In addition, the amount of BCIN used here was very small ($2.83 \times 10^{-5}$ mg/mm$^2$), so the color appearing on paper 3 was very weak.

Both papers 1 and 2 gave fast and visible response to samples, but paper 1 was darker (Figure 6-2 and 6-4) and displayed higher color intensity (Figure 6-5) than paper 2. Table 6-1 shows that double the amount of NBT was used to prepare paper 1. The reaction scheme on paper 1 and 2 was illustrated in Eqs. 6-1A and 6-1C. There is no need to change buffer pH. Indoxyl from the first step of 1A was oxidized by NBT under the same condition, which was catalyzed by the amine-containing membranes of PEI microcapsules. For the same amount of intermediate (halogenated indoxyl), its oxidized dimer (indigo dye) was enhanced by double amount of oxidizer of NBT. Paper 1 gave a noticeable response at 6 min, which is faster than the patent reported by Dwir et al. [3] They reported no optimal pH was found under which the overall reaction to yield the indigo dye proceeds fast enough to give a signal in less than 10 min. And their reported optimal pHs for two steps were 5.5-6 and ≥ 9 respectively, which was shown in Eqs. 6-

1A and 6-1B. The components of paper 1 per centimeter paper are listed in Table 6-2, BCIN used in paper 1 strips with the size of 2 mm×5 mm was much less than it in assembled card in Dwir's patent [3] with width of 4 mm ($2.83\times10^{-4}$ mg in our strip vs. $3.53\times10^{-2}$ mg in Dwir's patent), but response was faster than it, since this colorimetric reaction was catalyzed by PEI microcapsules (Eq. 6-1 C). Figure 6-3 shows the activity of paper 1 and 5 by adding of the same amount of neuraminidase. For the hydrolysis of BCIN, both papers did not have a significant difference, but paper 1 was getting darker than paper 5 (as expected by Eqs. 6-1A and 6-1B at pH 6) after 10 min, and showed remarkably darker color with increased length of reaction time. The same amount of BCIN and NBT were loaded onto both papers, but paper 1 contained microcapsules and paper 5 was prepared only by paper pulp. It was found that papers 1 and 5 displayed different color intensities in the presence of the same amount of sialidase (Figure 6-3), which indicated that the combination of microcapsules efficiently accelerated this color reaction. In practice, paper 1 has better visibility than paper 5 due to its uneven intensity generated by PEI microcapsules and their aggregrates containing high concentrations of BCIN and NBT. Paper 5 displayed uniform color, since it did not contain microcapsules, By comparison with all kinds of paper strips prepared, paper 1 showed the fastest response and most intense color.


A
BCIN
sialidase
pH =5.5-6.0
NANA
Indoxyl
(light yellow color)
[O]
pH > 9
Indigo dye
NANA =
B
pH > 9
(NBT)




Equation 6-. Reaction mechanism of chromogenic substrate of sialidase. Equation 6-1A: BCIN was hydrolyzed in the presence of sialidase at pH 5.5-6 to produce indoxyl, and then indoxyl isomers were oxidized to blue color indigo dye at higher pH (pH > 9); Equation 6-1B: indoxyl (hydrolyzed monomer) was oxidized in the presence of NBT at higher pH (pH > 9) to form a dark purple precipitate; Equation 6-1C: The redox reaction of indoxyl and NBT was catalyzed by secondary amino groups (on the membranes of PEI microcapsules) at the same pH.

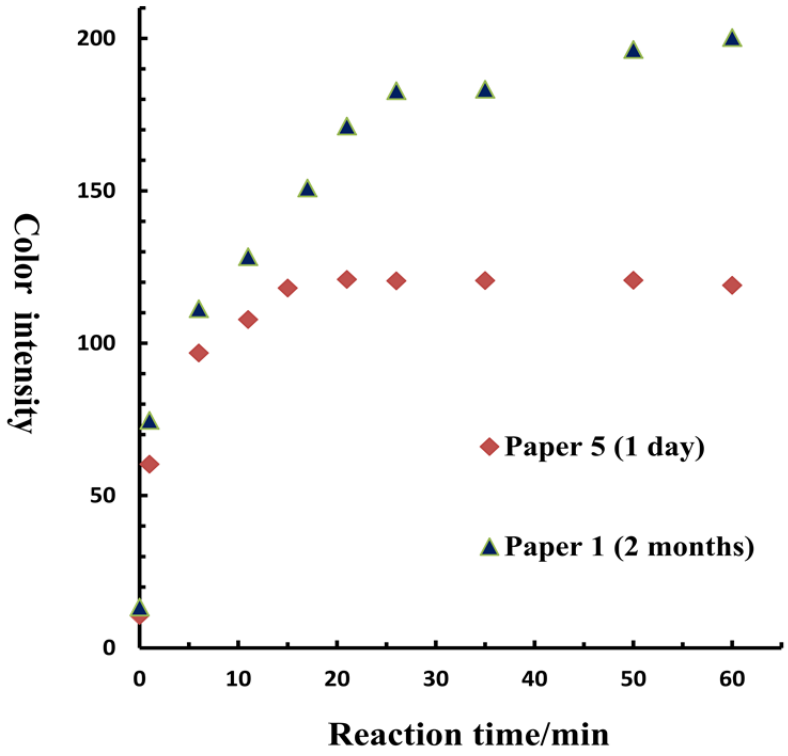



Figure 6- The color change of paper 1 and 5 with different length of reaction time in the presence of 15 µl (0.937 UN) sialidase. Paper 1 was measured after 2 months of preparation, and has faster and intense response than paper 5 (1 day) due to the incorporation of microcapsules.

NBT in papers 1 and 2 could react with the microcapsules to form a little bit of pink product at room temperature, which was shown in Figure 6-2 (papers 1 and 2 at 36 hours). But it was much slower than the enzymatic reaction. Therefore, it does not really affect detection of sialidase. The rest part on paper 3 and 4 did not change to pink after 36 hours, since NBT was not used for the fabrication of paper 3, and paper 4 did not contain microcapsules, so NBT was not retained in the paper after filtration.

Table 6-. Paper 1 components per square centimeter of paper.

| Microcapsules $g/cm^2$ paper | Wet pulp $g/cm^2$ paper | BCIN $mol/cm^2$ paper | NBT $mol/cm^2$ paper | Loading efficiency |
|---|---|---|---|---|
| 0.144 | 0.144 | $2.24\times10^{-8}$ | $5.10\times10^{-8}$ | Assume 100% |
| 0.144 | 0.144 | $5.28\times10^{-9}$ | NA | Measured, 22.2% |

### 6.4.3 The same paper with different amounts of sialidase

Papers 1 and 2 changed their color with different lengths of reaction time in the presence of various amount of sialidase (Figure 6-4). Paper 1 was measured after 55 days of preparation, and had a little bit faster response than paper 2 (12 days) due to the high amount of NBT. Papers 1 and 2 showed noticeable color change by adding of 2 µl (0.125UN) sialidase and a visible dark purple color appeared in the presence of 5 µl (0.312UN) sialidase. With the addition of 10 µl (0.625 UN) sialidase, both papers displayed significant dark purple color change.

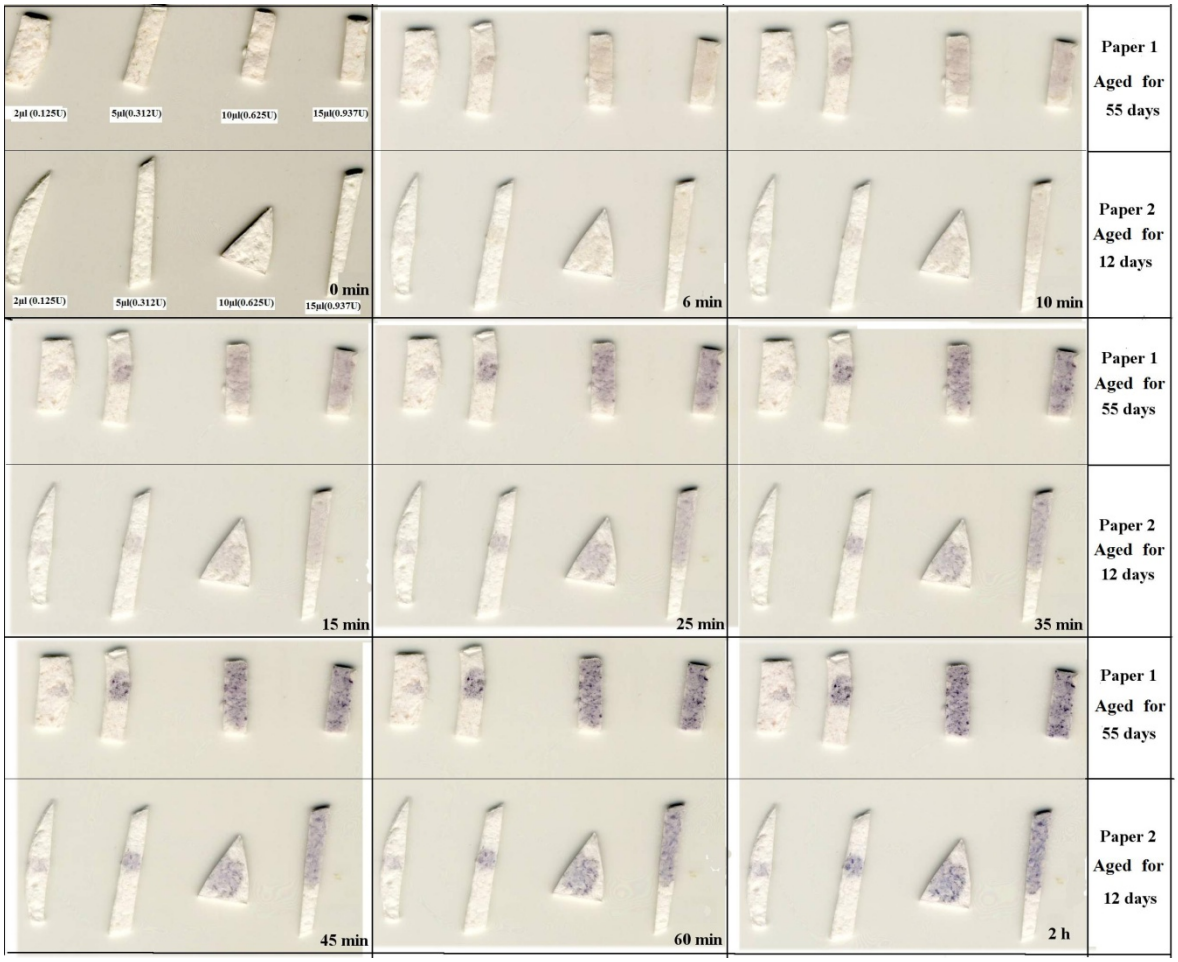



Figure 6- The color change of papers 1 and 2 with different lengths of reaction time in the presence of various amount of sialidase (2 µl, 5 µl, 10 µl, 15 µl). Paper 1 was measured after 55 days of preparation, and had faster response than paper 2 (12 days) due to the high amount of NBT. Both paper strips can give visible dark purple color response in the presence of 5 µl (0.312 UN) sialidase. The noticeable color was developed as fast as 6 min.

Figure 6-5 showed the intensity comparison of paper 1 and 2 in the presence of 5 µl and 10 µl sialidase at different time length, which clearly indicated paper 1 have higher

color intensity than paper 2 due to higher amount of color enhancer NBT. Paper 1 can give visible color in 6 min, which is faster than paper 2 (15 min).

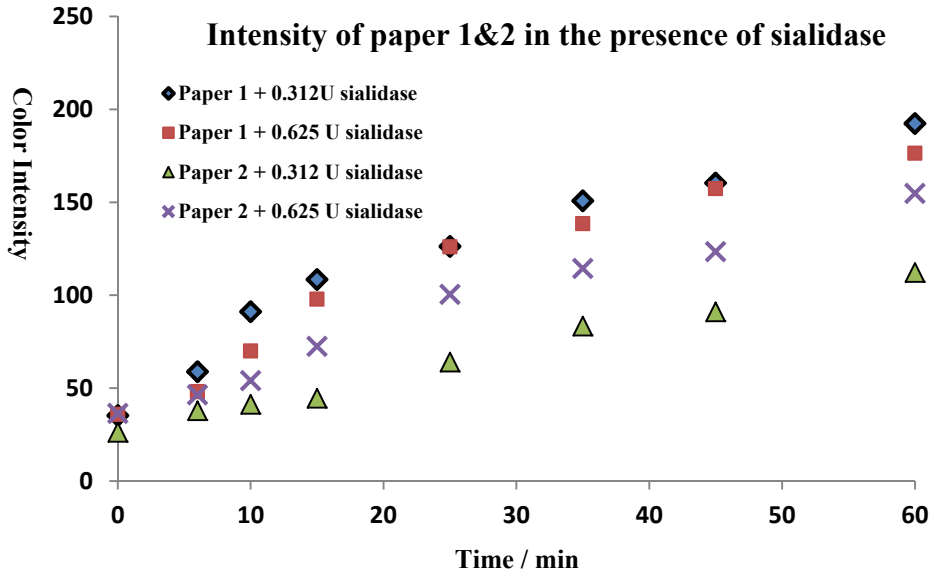



Figure 6- Mean color intensity of paper 1 and 2 in the presence of sialidase at different time length.

### 6.4.4 Loading efficiency of BCIN

The standard curve of BCIN is shown in Figure 6-6. The loading efficiency of BCIN was calculated to be 22.2%.

To date, paper 1 was kept for 13 months at -18°C and it still works well. A putative explanation for this is that PEI microcapsules can prolong the storage stability of BCIN.

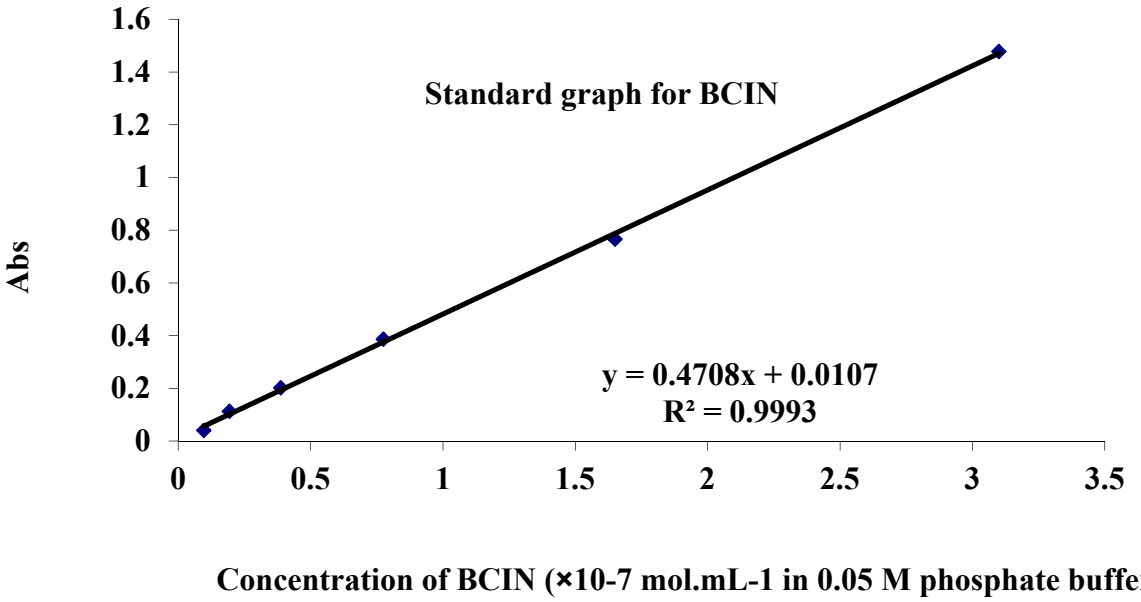



Figure 6- Standard graph of BCIN. The measurements were conducted by UV-Vis absorbance at wavelength of 280 nm. Original concentration of BCIN was used in paper preparation was $3.1\times10^{-7}$ mol.mL$^{-1}$ in 0.05M phosphate buffer. And other solutions were obtained from different dilutions.

## 6.5 Conclusions

A colorimetric biosensor for the detection of neuraminidase was successfully fabricated through incorporating PEI microcapsules containing BCIN and NBT into paper pulp. The portable paper strips could be easily stored at -18°C, since BCIN is sensitive to temperature. Paper strips kept the same activity over a minimum of 12 months. This colorimetric spot assay did not require any sample preparation step, and low amounts of sample (less than 10 µl) can be directly added to small pieces of the bioactive paper. The noticeable response time was in as little as 6 min, and the dark

purple color can be developed by increasing the reaction time. NBT and microcapsules enhanced the paper color by accelerating the second step of the reaction of BCIN and sialidase, which made it possible to have trace amounts of BCIN in paper but generate significant color response in the presence of sialidase. In practice, there is no expertise requirement for this simple one-step test, thus it could be used by women in the privacy of their home. Since the paper fabrication step is compatible with industrial application, this method could be potentially commercialized. Some further studies, such as paper response to different types of sialidase and real specimens from patients are envisioned in future work.

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# Chapter 7 Antibody sensitized microcapsules for the detection and deactivation of pathogens

## 7.1 Introduction

Antibody immobilization has become one of the most favorable approaches owing to its potential applications in biosensors for pathogen, fungus and virus detection [1-5], medical diagnostics [6-9], environmental monitoring [10, 11] and the food industry [12, 13]. Microencapsulation as a traditional immobilization method is now being applied in the preparation of immunosensors and biosensors by entrapment of biorecognition elements.

Antibody microcapsules can serve as a basis for immunosensor or biosensor-based analytical platforms in the detection of pathogens and their associated toxins by incorporating monoclonal, polyclonal or recombinant antibodies. Furthermore, microencapsulation has been studied as a means of avoiding interference from maternal antibodies; due to its protective capsules, microencapsulated reovirus can bypass suckled, neutralizing maternal antibodies that inhibit the active immunization of neonates [14]. Nonetheless, the most practical applications of microencapsulation protect antibodies against neutralization rather than viruses. Wallaces [15, 16] described a magnetic microencapsulated antibody displacement assay, in which semi-permeable nylon microcapsules were used to entrap antibodies for the detection of thyroxin in clinical practice. The addition of magnetic cellulose at the end of the incubation step

speeded up the sedimentation process producing a more stable pellet containing magnetic antibody-entrapped microcapsules. Vascular endothelium (VE)-cadherin-secreting hybridoma cells were also immobilized in alginate-agarose microcapsules to inhibit angiogenesis [8]. Hybridoma cells were protected within the biocompatible and semi-permeable membranes that permitted the exit of anti-VE-cadherin monoclonal antibodies but not the entry of cellular immune mediators. 1B5 hybridoma cells were grown within poly-L-lysine-coated alginate and agarose beads and secreted anti-VE-cadherin antibodies for 9 days of culture. The observed anti-angiogenic effect was antibody concentration-related, demonstrating a controlled drug delivery system. US patent 4483928 [17] reported a similar simple method to selectively measure the antigen of lymphocytes using a cell-mediated immunity approach where microcapsules are sensitized with the antibody to the antigen of lymphocyte. This simple strategy based on cell-mediated immunity comprises the quantification of microcapsules sensitized with antibodies against an antigen of lymphocyte and sheep red cells that are optically distinguishable from the antibody sensitized microcapsules.

Apart from antibody microcapsules, antigenic microcapsules were also used in clinical evaluations. Kobayashi [18] has performed passive agglutination tests using chemically stable polyurethane microcapsules sensitized with various antigens. A quantitative passive agglutination test of antibodies specific to *Treponema Pallidum* for the detection of serodiagnosis of syphilis was carried out with microcapsules instead of conventional sheep erythrocytes. The microcapsules used in this kind of study required

the following features: an absence of antigenic substance on the particle surface to avoid non-specific reactions, good chemical stability, the possibility of mass production with uniform quality and the modification of particle characteristics such as particle size, specific gravity and surface properties according to the purpose of the test.

Based on the success of prior studies, we attempted to encapsulate antibodies by PEI microcapsules. Given that the membranes of PEI microcapsules contain nitrogen, activated antibodies were likewise being conjugated to its membranes. The aim of this work was to develop a pathogen detection/deactivation platform. As we know encapsulated GOx can produce hydrogen peroxide in the presence of glucose, which can kill pathogens close to membranes, so GOx microcapsules have killing capacity. Since concentration of hydrogen peroxide produced from GOx microcapsules is low, we conjugate antibody to the membranes of microcapsules that can capture pathogens to improve their killing capacity. On the contrary, empty microcapsules do not have this killing ability, since they do not produce hydrogen peroxide.

## 7.2 Materials and methods

### 7.2.1 Materials

Empty PEI microcapsules and GOx microcapsules were previously synthesized [19], Rabbit polyclonal antibody, goat polyclonal to *Escherchia Coli* (ab25823), goat polyclonal to *Escherchia Coli* (Horseradish peroxidase). (ab69185), rabbit anti-goat IgG (labeled by FITC), and Rabbit F(ab')2 fragment to goat lgG (ab47846) were

purchased from Abcam Inc. Immunoglobulin G (from human plasma) was purchased from Athen Research and Technology. Sodium (metal) periodate ($NaIO_4$), sodium cyanoborohydride ($NaBH_3CN$), sodium sulfite ($Na_2SO_3$), ethanolamine ($HO(CH_2)_2NH_2$), poly(ethyleneimine) (Mw: 1300 Da), sebacoyl chloride, and glucose oxidase from *Aspergillus Niger* were purchased from Sigma-Aldrich. Miller's LB Broth used for propagation and maintenance of E.Coli, Difo™ Granulated Agar and Filterplate for rapid ELISA were purchased from Whatman Inc.

### 7.2.2 Methods

#### *7.2.2.1 Antibody microencapsulation*

Antibody microencapsulation followed the same protocol as for enzyme microcapsules. Microdroplets from the aqueous phase containing Immunoglobulin G from human plasma labeled by Texas-red and poly(ethyleneimine) labeled by FITC were crosslinked with sebacoyl chloride at the interface of microemulsion. The resulting microcapsules were first rinsed with cyclohexane to remove hydrophobic species followed by plenty of Milli-Q water to remove hydrophilic residues.

#### 7.2.2.2 *Conjugation of antibody to membranes of microcapsules*

This assay refers to the antibody-enzyme conjugates introduced by Hermanson [21]. In that the membranes of microcapsules contain amine groups like enzyme molecules, activated antibodies can be conjugated to its membranes. Many immunoglobulin

molecules are glycoproteins that can be periodate-oxidized to contain reactive aldehyde residues. Polyclonal IgG molecules often contain carbohydrate in the Fc portion of the molecule. This was removed from antigen binding sites to allow conjugation to take place through the polysaccharide chains without compromising its activities [21]. Antibody-microcapsule conjugation by reductive amination was accomplished by the oxidation of antibody and subsequent crosslinking to the amine-containing outer membranes. In this study empty microcapsules and GOx microcapsules were conjugated to goat anti-*E.coli* antibodies. The procedure of antibody conjugation to the empty and GOx microcapsules is given in Annex 1.

#### *7.2.2.3 Confirmation of antibody-microcapsule conjugation*

In order to confirm the conjugation between goat anti-*E.Coli* antibodies and microcapsules, three different assays were applied. The first assay used was confocal laser scanning microscope (CLSM). Ab-conjugated microcapsules were first blocked by skimmed milk to avoid unspecific binding and then incubated in secondary FITC-antibodies. If the primary antibodies (conjugated to microcapsules) were successfully conjugated to microcapsules and their binding sites were still active, the secondary FITC-labeled antibodies could be observed on the membranes of microcapsules by CLSM. The protocol is described in Annex 2.

The second assay was a traditional ELISA (see Annex 3). If the goat anti-*E.Coli* antibodies were well conjugated to microcapsules, they would bind to their antigens

(*E.Coli*) and the bound antigens could be detected by secondary antibody-HRP which would give off a blue color in the presence of 3, 3', 5, 5' –Tetramethylbenzidine (TMB) for ELISA. In practice, conventional ELISA for antibody-microcapsules presented challenges when binding the microcapsule-antibody conjugates to the microplates. The antibody-modified microcapsules tended to detach from the plate due to their large size and duo to the formation of microcapsule aggregates.

The third assay was a rapid ELISA (Annex 4). Rapid ELISA overcame the problems of conventional ELISA by using vacuum filtration of the microcapsules in the filter plate assay, avoiding the washing step and reducing the amount of time required.

#### *7.2.2.4 Enzymatic activity of GOx microcapsules-antibody*

Enzymatic activity measurements were conducted with an oxygen electrode (Rank Brothers Ltd.). Since the unconjugated GOx microcapsules and those conjugated with goat anti-*E.Coli* antibodies were suspended in buffer, membranes of microcapsules are solid. It was impossible to measure their absorbance with a traditional UV-Vis spectrophotometer. Given that GOx's reactions with glucose have consumed oxygen, their activities were measured in an oxygen cell chamber (3 ml total volume) with an oxygen electrode from Rank Brothers, which is sensitive to oxygen concentration. The oxygen consumption speed was considered as the relative enzymatic activities.

#### *7.2.2.5 The performance of GOx microcapsules conjugated with antibody*

Colony count estimation provides an inexpensive and user-friendly protocol for quantitative and qualitative bacterial pathogen detection and is routinely employed in the development of hazard analysis and critical control point (HACCP) systems in the food industry and in the establishment of risk assessment [22, 23].

In that glucose oxidase microcapsules can produce hydrogen peroxide ($H_2O_2$) in the presence of glucose, Goat anti-*E.Coli* antibodies conjugated to glucose oxidase microcapsules could capture E.Coli thereby killed by the hydrogen peroxide released. Thus, glucose oxidase microcapsules conjugated with antibodies would have the highest killing capacity. Empty microcapsules might absorb some E.Coli cells due to its porosity (less than 5 nm), since they are unable to produce hydrogen peroxide. Their killing capacity would be much lower than that of glucose oxidase microcapsules. Different microcapsules' suspensions were incubated with *E.Coli* cells to determine their killing capacity.

Various concentrations of capsule suspensions were diluted to $10^{-3}$ and $10^{-4}$ of original concentrations. 50 µl of each suspension of capsules in PBS 7.4 was added to 50 µl of *E.Coli* culture ($2\times10^7$ cells/ml) along with 15 µl of Glucose (1 M) and was incubated for 30 minutes at 37°C. The suspensions were diluted by PBS 7.4 to $10^{-3}$ or $10^{-4}$ of original concentrations and incubated in a Petri dish at 37°C overnight for colony counting.

## 7.3 Results and discussion

### 7.3.1 Activity of conjugated GOx microcapsules

Figure 7-1 shows the relative activities of GOx microcapsules and those of antibody conjugated GOx microcapsules. In Figure 7-1, a sharp slop means high activity. Antibody conjugated GOx microcapsules retained only 64% of its enzymatic activity. During the conjugation process, encapsulated GOx came into contact with many harsh chemicals such as sodium periodate, sodium cyanoborohydride, sodium sulfite and ethanolamine. Although the membranes of microcapsules offered some protection to the encapsulated GOx, they were nonetheless porous; if the process took too long, some activities would have been diminished.

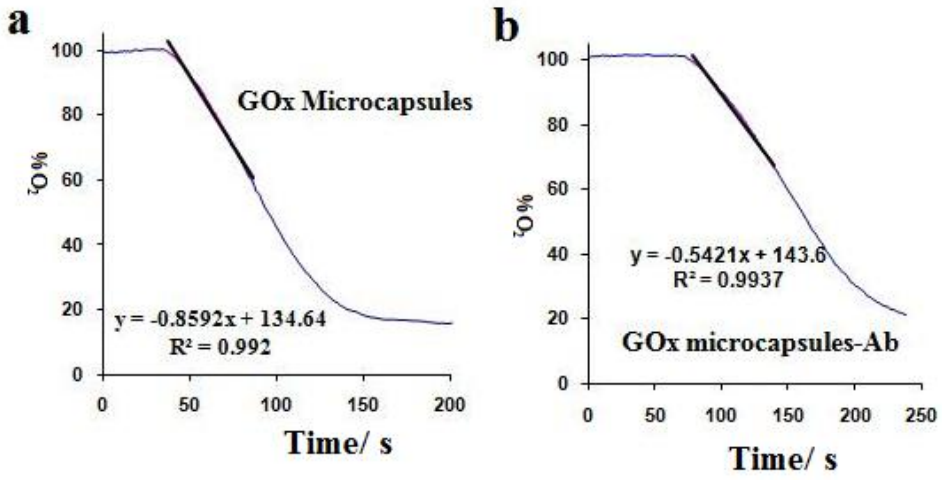


Figure 7- Plots of oxygen consumption speed as a function of enzymatic activity of GOx microcapsules (a) and Ab-GOx microcapsules (b), showing the relative activity.

### 7.3.2 CLSM study of antibody-microcapsule conjugation

CLSM suggests aggregation of both types of microcapsules, singe microcapsules could not be captured due to weak signal. Empty microcapsules without conjugation did not give a FITC signal or showed very weak green color. Figure 7-2, a is fluorescent image of b (transmitted light) illustrates the big aggregate of microcapsules giving off a weak fluorescence. The weak signal was probably due to unspecific adsorption of microcapsules' membranes to secondary antibodies. On the contrary, antibody-microcapsule- conjugates gave much stronger signals around the membranes because of the specific binding between antibody-microcapsules and secondary antibodies labeled with FITC. Figure 7-2 c shows a fluorescent image of microcapsules d, and image d was taken under transmitted light.

### 7.3.3 Conventional and rapid ELISA

The length of *E.Coli* was approximately 2 μm, but the pore size of the membrane of microcapsules was larger. Microcapsules could not be completely removed from the plate, thus rendering a strong background problematic. The issue was further complicated by the fact that porous GOx microcapsules could absorb *E.Coli* cells and the absorbed cells could bind goat anti-*E.Coli* antibodies (HRP), whereby the GOx microcapsules without antibody would also bind secondary antibody and give absorbance. However, GOx microcapsules conjugated with antibodies could bind more *E.Coli* and give a stronger absorbance. For conventional ELISA, GOx capsules-Ab

(0.242) showed higher absorbance than GOx capsules (0.124) using same kind of ashless paper with pore size 2.5 µm.

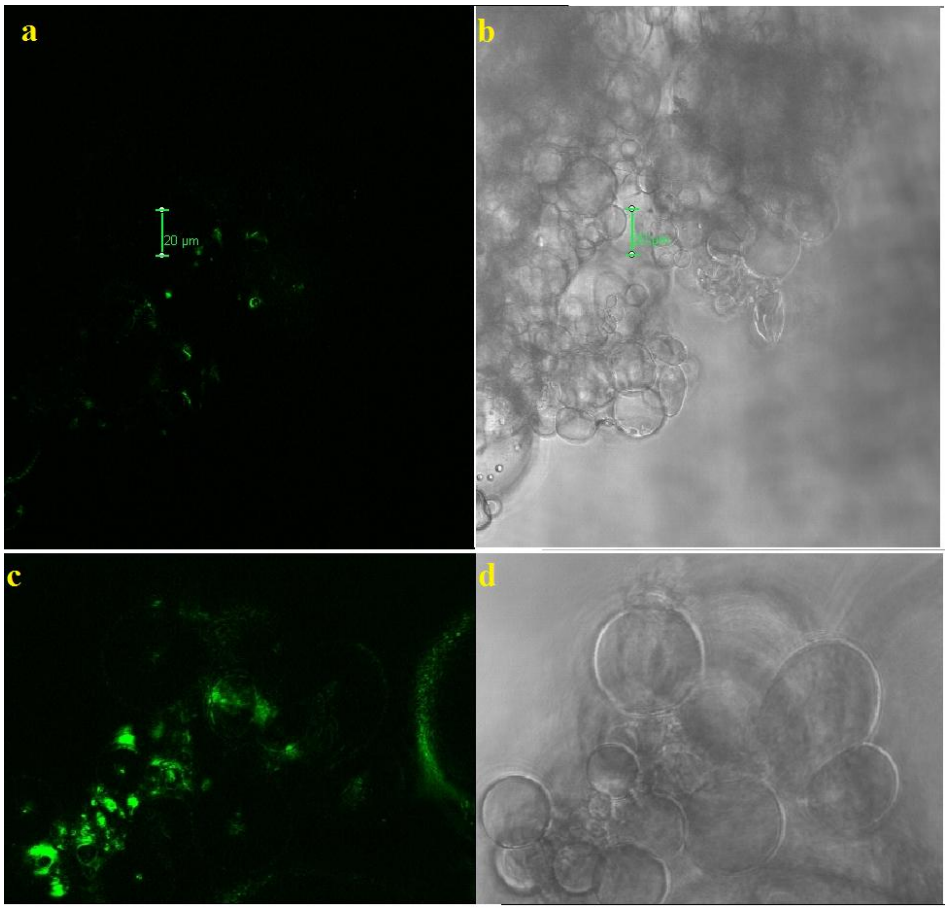


Figure 7- Confocal micrographs of Goat anti-*E.Coli antibody*-microcapsules (c, d) and empty microcapsules (a, b) incubated in rabbit anti-goat IgG, F(ab')2 fragment.

GOx microcapsule-antibody conjugates did not show a significant difference in absorbance compared to that of GOx microcapsules alone in rapid ELISA (Figure 7-3).

According to our tests on different filter papers, glass fibers as well as binding-free glass were not suitable for rapid ELISA due to strong absorbance.

### 7.3.4 Killing assay

Fifteen microliter of glucose solution was added to 50 µl of a GOx capsule suspension in PBS pH 7.4 and 50 µl of *E.Coli* culture ($2\times10^7$ cells/ml) and the mixture was allowed to react for 30 min at 37°C. GOx was diluted to different concentrations and the results are shown in Table 7-1. Colony-forming unit for each dilutions were compared to buffer incubation as positive control to get their killing capacities. The killing capacity of GOx microcapsules was 100% (colony-forming unit is 0) at concentration of 0.167 $mg.ml^{-1}$. At concentration of 0.0013 $mg.ml^{-1}$, microcapsules still exhibit more than 50% killing capacity. Since the microcapsules' suspension was not quite uniform in small volumes, different measurements might present some deviations.

The killing capacities of GOx microcapsules and GOx microcapsule-antibody conjugates were compared (Table 7-2). GOx microcapsules-Ab showed a slightly higher killing capacity than solely that of GOx microcapsules, suggesting that the killing capacity mainly derived from hydrogen peroxide produced by glucose oxidase. The performance of microcapsule-antibody conjugates was better with lower concentrations of microcapsules. At low concentrations of microcapsules, the antibodies effectively captured suspended *E.Coli* and thus the killing capacity of glucose oxidase with the microcapsules was improved.

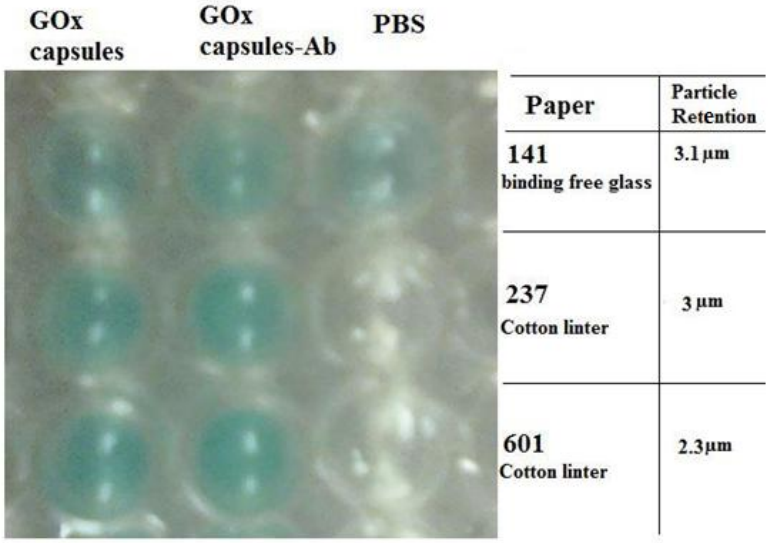


Figure 7- Rapid ELISA result for GOx microcapsules and GOx microcapsules-Ab.

Table 7- Killing capacity of GOx microcapsules with various dilutions

| Concentration of GOx/mg.ml$^{-1}$ | Dilution | CFU/100 µl | Killing/ % |
|---|---|---|---|
| 0.167 | $10^{-3}$ | 0 | 100 |
| | $10^{-4}$ | 0 | 100 |
| 0.0833 | $10^{-3}$ | 6 | 99.2 |
| | $10^{-4}$ | 1 | 97.4 |
| 0.0417 | $10^{-3}$ | 10 | 98.6 |
| | $10^{-4}$ | 0 | 100 |
| 0.0208 | $10^{-3}$ | 28 | 96.4 |
| | $10^{-4}$ | 6 | 84.2 |
| 0.0104 | $10^{-3}$ | 57 | 92.1 |
| | $10^{-4}$ | 7 | 81.6 |
| 0.0109 | $10^{-3}$ | 81 | 88.9 |
| | $10^{-4}$ | 16 | 57.9 |
| 0.0026 | $10^{-3}$ | 179 | 75.4 |
| | $10^{-4}$ | 17 | 55.3 |
| 0.0013 | $10^{-3}$ | 266 | 63.4 |
| | $10^{-4}$ | 19 | 50 |
| N.C. (PBS) | $10^{-3}$ | 726 | |
| | $10^{-4}$ | 38 | |

Table 7- Killing capacity of GOx microcapsules and GOx microcapsules-Ab

| Concentration of microcapsules/mg.ml$^{-1}$ | | Dilution | CFU/100 µl | Killing/ % |
|---|---|---|---|---|
| $1.3\times10^{-3}$ | GOx capsules | $10^{-3}$ | 130 | 63.9 |
| | | $10^{-4}$ | 16 | 42.8 |
| | GOx capsules-Ab | $10^{-3}$ | 116 | 67.8 |
| | | $10^{-4}$ | 17 | 39.3 |
| $6.5\times10^{-4}$ | GOx capsules | $10^{-3}$ | 171 | 52.5 |
| | | $10^{-4}$ | 16 | 42.8 |
| | GOx capsules-Ab | $10^{-3}$ | 144 | 60 |
| | | $10^{-4}$ | 17 | 39.3 |
| $3.3\times10^{-4}$ | GOx capsules | $10^{-3}$ | 177 | 50.8 |
| | | $10^{-4}$ | 21 | 25 |
| | GOx capsules-Ab | $10^{-3}$ | 180 | 50 |
| | | $10^{-4}$ | 25 | 10.7 |
| $1.6\times10^{-4}$ | GOx capsules | $10^{-3}$ | 198 | 45 |
| | | $10^{-4}$ | 24 | 14.3 |
| | GOx capsules-Ab | $10^{-3}$ | 255 | 29.2 |
| | | $10^{-4}$ | 25 | 10.7 |
| N.C. (PBS) | | $10^{-3}$ | 360 | |
| | | $10^{-4}$ | 28 | |

### 7.3.5 Microencapsulation of Immunoglobulin G from human plasma

IgG (from human plasma) labeled by Texas-red was encapsulated in PEI microcapsules tagged by FITC. The location of the encapsulated IgG upon

microencapsulation is illustrated in Figure 7-4. The green signal shown in (a) corresponds to the FITC-tagged membranes, the red signal in (b) is the core phase labeled by Texas-red and finally (c) is the overlay image of the membrane and the core. Most of the IgG was crosslinked in the membranes or bound to the internal walls of microcapsules; several were encapsulated within the core. The IgG can be protected by biocompatible and semi-permeable membranes that permit the exit of small molecules.

Antibodies were encapsulated into PEI microcapsules and the encapsulated antibodies were located in the cores or fixed into the membranes of microcapsules. Antibodies were also conjugated to the outer membranes of microcapsules by reductive amination. After conjugation, GOx microcapsules had lost 36% of enzymatic activity due to the interaction with harsh chemicals used during the conjugation process. Our studies with CLSM, ELISA and *E.coli* killing assay demonstrate that goat-anti-*E.Coli* antibodies were successfully conjugated to the microcapsules. Nevertheless, the concentrations of antibodies are low and thus the binding performance of the antibodies was weak. Both GOx microcapsules and GOx microcapsule-antibody conjugates had killing capacities as GOx produced hydrogen peroxide in the presence of glucose. Results suggest that the killing capacity of GOx microcapsule-antibody conjugates was almost the same as that of GOx microcapsules at a relative high concentration of the microcapsules' suspension, though it had a slightly higher killing capacity at a lower concentration of suspension. This was explained as a more efficient

capture of *E.coli* by the antibody on the outer membranes enabling higher killing capacity of glucose oxidase within microcapsules.

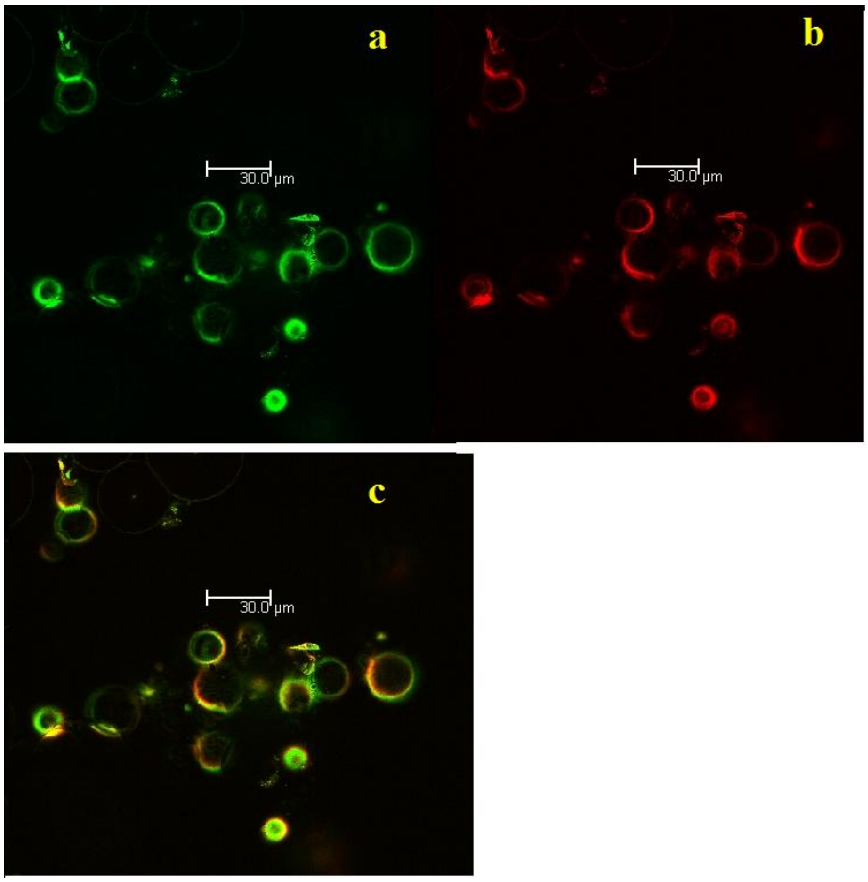


Figure 7- Multi-panel view of confocal images of IgG microcapsules. PEI membrane-FITC signal (a), IgG human plasma of core phase-Texas-red signal (b), the overlay image of the core and the membrane of microcapsules (c).

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# Chapter 8 Conclusions and future work

## 8.1 Conclusions

We have successfully fabricated PEI microcapsules by the emulsion method and the vibration nozzle method and have compared both methods. A method based on laminar jet break-up technique using a commercial instrument developed to produce alginate beads is reported for the first time for production of PEI microcapsules. Optimal parameters for both methods were studied and manufactured microcapsules were characterized. Some enzymes such as TvL and GOx were encapsulated by both methods. By comparison, the emulsion method was chosen for enzyme microencapsulation due to its higher encapsulation efficiency, higher productivity, lower toxicity and controllable sizes and size distribution. Depending on the properties of microcapsules, enzyme microcapsules can be immobilized in paper by different ways including coating, sizing and printing.

We firstly demonstrated the interaction between PEI and laccase. The enzymatic activities of free and encapsulated enzymes were compared, and the factors affecting enzyme activities were discussed. A simple mixing assay, a fluorescence assay, a circular dichroism assay and kinetic studies were carried out for interaction measurements. The results revealed that TvL can interact with the PEI molecules; TvL's conformation was changed and its activity was decreased by the interaction between Cu in enzyme's active sites and amino groups in PEI molecules. However, no interaction was found between GOx and PEI molecules or all the interactions

contributions were eliminated. Therefore, we determined that PEI does not change activity of the GOx. The decrease in GOx's activity after microencapsulation was due to the closed shells with pore size less than 5 nm. Some other factors, such as reduction of the amount of oxygen inside microcapsules (substrate depletion) can also reduce it activity.

Two kinds of bioactive paper fabrication platform based on microencapsulation were built up either through incorporating microencapsulated enzymes into paper pulp or incorporating empty PEI microcapsules socked in BCIN and NBT into paper pulp in the making. Both methods are scalable. We successfully fabricated two types of colorimetric bioactive paper strips named GOx paper and BV paper, which are inexpensive, sensitive and one-step spot assays. GOx paper containing GOx microcapsules changes color in the presence of glucose and KI, which can be potentially used for food packaging paper to improve shell life of food by scavenging oxygen that might penetrate the physical barrier.

The BV paper strip displays a dark-purple color in the presence of neuraminidase. Numerous studies have shown that bacterial vaginosis is associated with elevated sialidase. This colorimetric biosensor can detect the presence of sialidase for the diagnosis of bacteria vaginosis. The detection is as soon as 6 min. comparing to other techniques, BV paper strip is affordable, fast response, one-step spot assay with good storage stability, which was not discussed in previous publications. Comparing with commercial BVBlue test kit and Dwir's patent, this paper strip did not require to change buffer during detection and has better visibility. Patients can self-test at home

by BV paper strip before visiting a physician's office. Present BV paper strip was only tested by specific neuraminidase instead of real specimen, further study is required.

Preliminary antibody sensitized microcapsules were prepared by two approaches: the conventional encapsulation method like enzyme microencapsulation and the conjugation of the antibodies to outer membranes of microcapsules. The overall difference in performance of the two was that antibody was crosslinked in the membranes or inside of microcapsules' cores by encapsulation method and may lose some of activity duo to diffusion restriction structure changes in membranes. This study is only ground work, but some protocols were set up for this project.

## 8.2 Future work

### 8.2.1 Exploring different applications for PEI microcapsules

PEI microcapsules are sturdy and can be fabricated under mild conditions within short time period at low cost. Their characterization can be adjusted by changing a few experimental parameters, and have been well studied in previous work, demonstrating their potential in many applications.

Encapsulation protects antibodies from being neutralized during their detection and deactivation of pathogens. The challenge at this point is to maintain the activities of antibodies. PEI-SC microencapsulation is a matured technology in our group, but PEI microcapsules were mainly used for enzyme microencapsulation. Therefore, stability studies should be performed to determine the suitable parameters for antibody

microencapsulation. Structure of an antibody is related to its immune function, which might be affected by microencapsulation, since some molecules were crosslinked into membrane and antigen binding sites might be changed. Microcapsules must be biocompatible and semi-permeable, allowing the exit of antigens but not entry of antibodies. This can be accomplished by attaching antibodies on the surface of enzyme microcapsules by methods like conjugation to maintain their activity.

We have successfully fabricated colorimetric biosensors, for example, the glucose oxidase bioactive paper. In the future study, we could encapsulate other kinds of enzymes that response to different substrates. Microencapsulation protects vulnerable biological molecules from unwanted environmental factors for the fabrication of colorimetric biosensors.

PEI microcapsules are not soluble in water, which permits the absorption of impurities due to porous membranes, and they can be potentially used for water purification.

PEI microcapsules are biocompatible. The pore sizes of their membranes can be controlled by adjusting some reaction parameters during the fabrication process. The flexibility in their pore sizes allows in controlled release for chemicals.

8.2.2 Different microencapsulation methods have to be applied for different active agents.

Due to the interaction of amine groups in PEI with laccase either active site's copper or amino groups in its vicinity, laccase lost activity to a large extent after microencapsulation. Some other microencapsulation methods such as the use of various kinds of alginate beads, layer-by-layer technology, extrusion, spray drying, coacervation and so-gel process can be tested to improve activity of microencapsulated enzyme.

8.2.3 Optimization of paper-making method

Currently, bioactive paper is fabricated by filtration of paper pulp suspension containing enzyme microcapsules or empty microcapsules socked in active agents through Buchner funnel. The fabrication process can be optimized by changing some parameters such as the water and microcapsule contents in pulp suspension. The fabrication procedure is industrially compatible with paper-making process and can be up-scaled. Furthermore, the paper-drying process also can be optimized to improve the enzyme activity, some methods like lyophilization can be tried to dry sensitive paper that can maintain its activity. For example the BV paper strip contain BCIN that is light and temperature sensitive and the whole paper-making process must be protected from light under controlled temperature, followed by drying by lyophilization. In a large scale setting, different methods such as instant ultrahigh temperature, air-drying or

nitrogen-drying can be evaluated. Other methods of bioactive paper making such as coating or printing of microencapsulated active ingredients can be tried.

The most expensive agent used for BV paper strip is BCIN, which acts as substrate. Although Nitro blue tetrazolium (NBT) is an inexpensive agent, for large scale application, replacement with enhancers of even lower cost like other indigo dyes might be preferred. The ratio between BCIN and NBT should be optimized to improve sensitivity and minimize the cost.

As we reported, the BV paper is sensitive to neuraminidase from *Clostridium perfringens* (C. Welchii, TypeV). However, measurements on real specimens require the use of proper positive and negative controls or a pre-determined BCIN concentration that can detect sialidase and give a correct signal.

Collaboration with hospitals to facilitate access to real specimens is needed and a comparison of BV paper with traditional methods, such as Amsel criteria, Nugent score, Whiff test. Amsel criteria are the gold standard for the diagnosis of bacterial vaginosis. At least three of four criteria should be confirmed diagnosis. A characteristic fishy odor on wet mount is considered a positive whiff test and is suggestive of bacterial vaginosis [1].

The BV paper has only been tested with neuraminidase from *Clostridium perfringens* (C. Welchii, TypeV). The sensitivity of BV paper to different neuraminidases for example NEU3 should be assessed in future works.

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# Annexes

## Annex . The procedure of antibody conjugation to microcapsules.

Step 1 Antibody oxidation by reductive amination:

1. 20 μl Goat anti-E.Coli is diluted to 2 ml by 0.01M PBS in 2 ml eppendorf tube;

2. Add 100 μl $NaIO_4$ of 0.1 M, and react 20 min;

3. Add 1.74 mg of $Na_2SO_3$;

4. Centrifuge solution by MWCO10,000, and recover by pH 9.6 of 0.2 M carbonated buffer.

Step 2 Conjugation of oxidized antibody to the membranes of microcapsules:

1. Weight 2 g capsules rinsed by pH 9.6 of carbonated buffer, and add 4 mL pH 9.6 buffer and oxidized antibody; react for 2h;

2. Add 40 μl $NaBH_3CN$, react for 30 min;

3. Add 200 μl $NH_2CH_2CH_2OH$, react for 30 min;

4. Filtrate and rinse them by pH 7 of 0.01 M PBS.

## Annex . Confocal laser scanning microscope (CLSM) for the detection of conjugation.

a) Prepare 2% (w/v) commercial skimmed milk powder solution in water, and filter to remove particle;

b) Incubate antibody conjugated capsules in 1.5 ml of milk solution in 2 ml eppendorf microcentrifuge tube for 1h;

c) Rinse microcapsules. Remove all the supernatant, then rinse with PBS buffer and repeat centrifuge and supernatant removal step for 3 times.

d) Incubate antibody-capsules in 0.05 μg/ml secondary antibody of rabbit anti-goat IgG, F(ab')2 fragment coupled to FITC in PBS for 1h;

e) Remove unbound secondary rabbit anti-goat IgG, F(ab')2 fragment coupled to FITC from microcapsules by rinsing step described in step c for 5 rinsing cycles;

f) Carry out steps b-e with empty microcapsules;

g) Make sure there is no unspecific adsorption of secondary antibody on the capsules;

h) Observe them by CLSM .

## Annex . Conventional ELISA for the detection of conjugation.

Conventional ELISA is performed in plastic microplates.

a) 100 µl of E.Coli: DH5α is coated to 96-well plate, and incubate overnight at 4°C;

b) Rinse plate with 200 µl PBS for 5 times;

c) Block active sites of plate with 200 µl of 4% skim milk for 3 hours;

d) Rinse plate by 240 µl PBST (with 0.05% Tween 20) for 5 times;

e) Add 200 µl primary antibody-HRP and orbital shake for 1 hour;

f) Rinse with PBST for 5 times;

g) Add 100 µl secondary antibody-HRP and shake for 1 hour;

h) Wash them by PBST for 5 times;

i) Add 100 µl of TMB for ELISA and wait for blue color;

j) Stop them by $H_2SO_4$ (40 ml concentrated $H_2SO_4$ in 360 ml water), blue color will turn to yellow;

k) Read plate at 450 nm on microplate manager (Model 680) from Bio-Rad Laboratories, Inc.

## Annex . Rapid ELISA for the detection of conjugation.

a) Rinse filtration membranes, and stick on the silicon membrane. Cover removable top;

b) Add 200 µl PBS for 2 min to incubate membranes;

c) Add 100 µl 0.33 $mg.ml^{-1}$ microcapsules for 5 min;

d) Blocking active sites by 200µl of superblock® blocking buffer in TBS for 15 min;

e) Wash by 200 µl PBST for three times, drain by vacuum;

f) Add 100 µl of $10^8$ $cells.ml^{-1}$ DH5α ($10^7$ cells);

g) Wash twice by 200 µl PBST and drain by vacuum;

h) Add 100 µl goat anti-E.Coli-HRP with dilution of 1/200 ($8.5\times10^{-5}$), and incubated for 15 min. Drain by vacuum;

i) Add 100 µl of TMB for ELISA, and then wait for the color development;

j) Stop by $H_2SO_4$ (40 ml $H_2SO_4$ + 360 ml $H_2O$).

k) Negative control: microcapsules were instead of PBS.